

**DIVISION - CONTINUATION - CONTINUATION-IN-PART
APPLICATION TRANSMITTAL FORM**

Attorney Docket No.: A-460A

Anticipated Classification Of This Application:
Class Subclass

Prior Application:
Examiner

Art Unit

to the Assistant Commissioner for Patents:

This is a request for filing a ☐ continuation ☐ divisional ☒ continuation-in-part application, under 37 CFR 1.53(b), of pending prior application Serial No. 09/178,292 filed on October 23, 19 98, of Joan C. Egrie, et al.

for METHODS AND COMPOSITIONS FOR THE PREVENTION AND TREATMENT OF ANEMIA

For CONTINUATION or DIVISIONAL APPLNS only: The entire disclosure of the prior application, from which an oath or declaration is supplied under Box 1b, below, is considered a part of the disclosure of the accompanying continuation or divisional application and is hereby incorporated by reference. The incorporation can only be relied upon when a portion has been inadvertently omitted from the submitted application parts.

1. ☒ Transmitted herewith are:
- ☒ 57 pages of specification, 7 pages of claim(s) and 1 page of abstract, **totaling** 65 pages.
 - ☒ 12 sheet(s) of drawings.
 - ☒ 2 pages of **Unsigned** Oath or Declaration by the applicant(s):
 - ☐ a. Newly executed (original or copy)
 - ☐ b. Copy from a prior application (37 CFR 1.63(d)) (for continuation/divisional applns. only)
 - ☐ _____ pages of Sequence Listing; computer readable copy (identical to paper copy); sequence statement.

2. ☒ The filing fee is calculated below:

For	Number Filed		Number Extra		Rate		Fee
Total Claims	131	- 20 =	111	x	\$18.00	=	\$1,998.00
Independent Claims	5	- 3 =	2	x	\$78.00	=	156.00
Multiple Dependent Claims	27			+	\$260.00	=	260.00
Basic Fee					\$690.00	=	690.00
Total Filing Fee							\$3,104.00

3. ☒ Please charge Deposit Account No. 01-0519, in the name of Amgen Inc., in the amount of \$3,104.00. An original and one copy are enclosed.
4. ☒ Throughout the prosecution of this application, if any extension of time is necessary, please consider this a request therefor.
5. ☒ The Commissioner is hereby authorized to charge any additional filing fees which may be required by the accompanying application, any additional fees which may be required during pendency of this application as required by 37 CFR 1.16 or 1.17, or credit any overpayment to Deposit Account No. 01-0519 throughout the prosecution of this application.
6. ☐ Cancel in this application original claims _____ of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

EXPRESS MAIL CERTIFICATE

"Express Mail" mail labeling number: EL381977415US

Date of Deposit: April 21, 2000

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.16 on the date indicated above and is addressed to Box Patent Application, Assistant Commissioner for Patents, Washington, DC 20231

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7. ☒ Preliminarily, please amend the specification by inserting before the first line the following:

--This application is a ☒ continuation-in-part ☐ division of application Serial No. 09/178,292, filed October 23, 1998, which is hereby incorporated by reference.--

8. ☐ The prior application is hereby expressly abandoned as of the filing date accorded this application. A duplicate copy of this sheet is enclosed for filing in the prior application file. (May only be used if signed by person authorized by § 1.138 and before payment of base issue fee.)

8a. ☐ New formal drawings are enclosed.

9. ☐ Priority of application Serial No. _____ filed on _____ in _____ (country) is claimed under 35 USC 119.

9a. ☐ The certified copy has been filed in prior application Serial No. _____ filed _____

10. ☐ The prior application is assigned of record to _____

11. ☐ A preliminary amendment is enclosed.

12. ☒ Also enclosed Extension of Time to Respond in parent application

13. ☐ Other: _____

14. ☐ The power of attorney in the prior application is to: _____

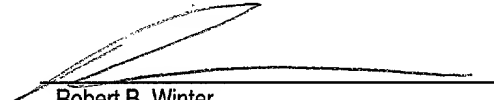
a. ☐ The power appears in the original papers in the prior application.

b. ☐ Since the power does not appear in the original papers, a copy of the power in the prior application is enclosed.

c. ☐ Address all future communications to _____
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**METHODS AND COMPOSITIONS FOR THE PREVENTION AND
TREATMENT OF ANEMIA**

5 Field of the Invention

 The invention relates to increasing
hematocrit in a mammal using hyperglycosylated analogs
of erythropoietin. More particularly, the invention
relates to less frequent dosing of a hyperglycosylated
10 analog compared to recombinant human erythropoietin to
raise and maintain hematocrit and treat anemia. The
invention also relates to administration of lower
amounts of a hyperglycosylated analog compared to
recombinant human erythropoietin at an equivalent
15 dosing frequency in order to raise and maintain
hematocrit and treat anemia. New hyperglycosylated
analogues of erythropoietin are also provided.

Background of the Invention

20 Erythropoietin (Epo) is a glycoprotein
hormone necessary for the maturation of erythroid
progenitor cells into erythrocytes. It is produced in
the kidney and is essential in regulating levels of red
blood cells in the circulation. Conditions marked by
25 low levels of tissue oxygen signal increased production
of Epo, which in turn stimulates erythropoiesis. A
loss of kidney function as is seen in chronic renal
failure (CRF), for example, typically results in
decreased production of Epo and a concomitant reduction
30 in red blood cells.

 Human urinary Epo was purified by Miyake et
al. (J. Biol. Chem. 252, 5558 (1977)) from patients
with aplastic anemia. However, the amount of purified
Epo protein obtained from this source was insufficient
35 for therapeutic applications. The identification and
cloning of the gene encoding human Epo and expression

of recombinant protein was disclosed in U.S. Patent No. 4,703,008 to Lin, the disclosure of which is incorporated herein by reference. A method for purification of recombinant human erythropoietin from cell medium is disclosed in U.S. Patent No. 4,667,016 to Lai et. al., which is incorporated herein by reference. The production of biologically active Epo from mammalian host cells has made available, for the first time, quantities of Epo suitable for therapeutic applications. In addition, knowledge of the gene sequence and the increased availability of purified protein has led to a better understanding of the mode of action of this protein.

Both human urinary derived Epo (Miyake et al. supra) and recombinant human Epo expressed in mammalian cells contain three N-linked and one O-linked oligosaccharide chains which together comprise about 40% of the total molecular weight of the glycoprotein. N-linked glycosylation occurs at asparagine residues located at positions 24, 38 and 83 while O-linked glycosylation occurs at a serine residue located at position 126 (Lai et al. J. Biol. Chem. 261, 3116 (1986); Broudy et al. Arch. Biochem. Biophys. 265, 329 (1988)). The oligosaccharide chains have been shown to be modified with terminal sialic acid residues with N-linked chains typically having up to four sialic acids per chain and O-linked chains having up to two sialic acids. An Epo polypeptide may therefore accommodate up to a total of 14 sialic acids.

Various studies have shown that alterations of Epo carbohydrate chains can affect biological activity. In one study, however, the removal of N-linked or O-linked oligosaccharide chains singly or together by mutagenesis of asparagine or serine residues that are glycosylation sites sharply reduces in vitro activity of the altered Epo that is produced

in mammalian cells (Dube et al. J. Biol. Chem. 263,
17516 (1988)). However, DeLorme et al. (Biochemistry
31, 9871-9876 (1992)) reported that removal of N-linked
glycosylation sites in Epo reduced in vivo but not in
5 vitro biological activity.

The relationship between the sialic acid
content of Epo and in vivo biological activity was
disclosed by determining the in vivo activity of
isolated Epo isoforms. It was found that a stepwise
10 increase in sialic acid content per Epo molecule gave a
corresponding stepwise increase in in vivo biological
activity as measured by the ability of equimolar
concentrations of isolated Epo isoforms to raise the
hematocrit of normal mice (Egrie et al. Glycoconjugate
15 J. 10, 263 (1993)). Those Epo isoforms having higher
sialic acid content also exhibited a longer serum half-
life but decreased affinity for the Epo receptor,
suggesting that serum half-life is an important
determinant of in vivo biological activity.

20 Introduction of new glycosylation sites in
the Epo polypeptide can result in the production of
molecules with additional carbohydrate chains. See PCT
Publication Nos. WO91/05867 and WO94/09257 hereby
incorporated by reference in their entirety. Epo
25 glycosylation analogs having at least one additional
N-linked carbohydrate chain and/or having at least one
additional O-linked carbohydrate chain are disclosed. A
glycosylation analog having one additional N-linked
chain was determined to have a longer circulating
30 half-life compared to recombinant human Epo (rHuEpo)
(isoforms 9-14) and to a purified isoform of rHuEpo
having 14 sialic acids per molecule.

Administration of recombinant human
erythropoietin (rHuEpo) is effective in raising red
35 blood cell levels in anemic patients with end stage
renal disease (Eschbach et al. New Eng. J. Med. 316,

73-38 (1987)). Subsequent studies have shown that treatment with rHuEpo can correct anemia associated with a variety of other conditions. (Fischl et al. New Eng. J. Med. 322, 1488-1493 (1990); Laupacis, Lancet 341, 1228-1232 (1993). Regulatory approvals have been given for the use of rHuEpo in the treatment of anemia associated with CRF, anemia related to therapy with AZT (zidovudine) in HIV-infected patients, anemia in patients with non-myeloid malignancies receiving chemotherapy, and anemia in patients undergoing surgery to reduce the need of allogenic blood transfusions. Current therapy for all approved indications (except the surgery indication) involves a starting dose of between 50-150 Units/kg three times per week (TIW) administered either by an intravenous (IV) or subcutaneous (SC) injection to reach a suggested target hematocrit range. For the surgery indication, rHuEpo is administered every day 10 days prior to surgery, on the day of surgery, and four days thereafter (EPOGEN® Package Insert, 12/23/96). In general, the current recommended starting doses for rHuEpo raise hematocrit into the target range in about six to eight weeks. Once the target hematocrit range has been achieved, a maintenance dosing schedule is established which will vary depending upon the patient, but is typically three times per week for anemic patients with CRF. The administration of rHuEpo described above is an effective and well-tolerated regimen for the treatment of anemia.

It would be desirable to have a therapeutic with greater potency than rHuEpo. An advantage to such a molecule would be that it could be administered less frequently and/or at a lower dose. Current treatments for patients suffering from anemia call for administration of EPOGEN® three times per week and for

5 surgery patients administration once per day. A less frequent dosing schedule would be more convenient to both physicians and patients, especially those patients who do not make regularly scheduled visits to doctor's offices or clinics, or those who self-inject their Epo. Another advantage of a more potent molecule is that less drug is being introduced into patients for a comparable increase in hematocrit.

10 It is therefore an object of the invention to identify more potent molecules for the treatment of anemia which will permit a less frequent dosing schedule. It is a further object of the invention to provide molecules which will increase and maintain hematocrit at levels which are at least comparable to
15 that of Epo when administered at a lower dose. It is also an object of the invention that these molecules selected for less frequent dosing is at least as well tolerated as rHuEpo and potentially better tolerated in some patients.

20

Summary of the Invention

It has been found that a hyperglycosylated Epo analog designated N47 (Asn³⁰Thr³²Val⁸⁷Asn⁸⁸Thr⁹⁰ Epo) has a longer serum half-life than recombinant human erythropoietin (rHuEpo) and a greater in vivo activity
25 when administered at the same dose and frequency as rHuEpo. Further, the analog has been shown to raise hematocrit in mice at once per week administration that is comparable to hematocrit rise for rHuEpo administered three times per week. The
30 pharmacokinetics of Epo analog N47 administered to mice and to humans were similar.

The invention provides for a method of raising and maintaining hematocrit in a mammal
35 comprising administering a therapeutically effective amount of an Epo hyperglycosylated analog in a

pharmaceutical composition, wherein the analog is administered less frequently than an equivalent molar amount of rHuEpo to obtain a comparable target hematocrit. The dosing frequency of the present invention in order to reach a patient's optimal hematocrit range is less than three times per week. Dosing frequencies may be two times per week, one time per week, or less than one time per week, such as one time every other week, once per month or once every two months. The dosing frequency required to maintain a patient's target hematocrit is less than three times per week. Dosing frequencies may be two times per week, one time per week, or less than one time per week, such as one time every two weeks, once per month or once every two months.

The invention also provides for a method of raising and maintaining hematocrit in a mammal comprising administering a therapeutically effective amount of an Epo hyperglycosylated analog wherein the analog is administered at a lower molar amount than rHuEpo to obtain a comparable target hematocrit.

Also provided for are pharmaceutical compositions comprising Epo hyperglycosylated analogs wherein the compositions are suitable for dosing frequency of less than three times per week. The compositions will include pharmaceutically acceptable adjuvants suitable for use with Epo hyperglycosylated analogs.

The invention may be employed with any condition resulting in a decrease in red blood cell levels, such as anemia associated with a decline or loss of kidney function, (chronic renal failure) myelosuppressive therapy, cancer, viral infection, chronic disease and excessive loss of blood during surgical procedures. In one embodiment, treatment with

once per week dosing, or less frequently, is for anemia resulting from chronic renal failure.

Also provided for are new hyperglycosylated analogs of Epo. The analogs comprise at least one
5 additional carbohydrate chain compared to rHuEpo wherein at least one N-linked carbohydrate chain is added at any of positions 52, 53, 55, 86 and 114. New hyperglycosylated analogs may have two, three or four additional carbohydrate chains, or may have more than
10 four additional chains.

Description of the Figures

Figure 1 shows the amino acid sequence of human erythropoietin.
15

Figure 2 shows a Western blot analysis of rHuEpo and Epo hyperglycosylated analogs from CHO cell expression in serum free medium. Construction of analogs N53 and N61 are described in Example 1. The
20 number of N-linked carbohydrate chains on each analog is indicated.

Figure 3 compares the activity of rHuEpo, Epo analogs N4, N18, and N50 (containing four N-linked
25 carbohydrate chains), N47 (containing five N-linked carbohydrate chains), and N53 (containing six N-linked carbohydrate chains) in the exhypoxic polycythemic mouse bioassay. Experimental procedures are described in Example 3. Each point represents the mean response
30 of five animals. Analogs N4, N18 and N47 have been described previously in W094/09257.

Figure 4 compares the serum half-life of rHuEpo and Epo analog N47 administered to normal rats
35 by intravenous injection (IV). Experimental procedures

are described in Example 4. Results are the mean (\pm SD) for each group.

Figure 5 compares the serum half-life of rHuEpo and Epo analog N47 administered to Beagle dogs by intravenous injection (IV). Experimental procedures are described in Example 4. Results are the mean (\pm SD) for each group.

Figure 6 shows the increase in hematocrit in mice in response to varying doses of rHuEpo or Epo analog N47 administered by intraperitoneal injection (IP) three times per week (TIW) for six weeks. Experimental procedures are described in Example 5. Results shown are the group mean (\pm SD) of the change in hematocrit for each dose group.

Figure 7 compares the relative potency in mice of rHuEpo and Epo analog N47 injected by either the intraperitoneal (IP) or intravenous (IV) routes of administration at a frequency of once weekly (QW) or three time a week (TIW). Experimental procedures are described in Example 5. Each point represents the mean (\pm SD) of data from separate experiments as follows:
N47, IP, TIW (n=5); N47, IV, TIW (n=1); N47, IP, QW (n=2); N47, IV, QW (n=3); rHuEpo, IP, TIW (n=5); rHuEpo, IV, QW (n=2). Each experiment used 7 - 13 mice per dose.

Figure 8 shows the increase in hematocrit in mice in response to varying doses of rHuEpo or Epo analog N47 administered by intravenous (IV) injection one time per week (QW) for approximately six weeks. Experimental procedures are described in Example 5. Results shown are the group mean (\pm SD) of the change in hematocrit for each dose group.

Figure 9 shows the increase in hematocrit in mice in response to varying doses of Epo analog N47 administered by intravenous (IV) injection one time per week (QW) or once every other week (EOW) for approximately six weeks. Experimental procedures are described in Example 5. Results shown are the group mean (\pm SD) of the change in hematocrit for each dose group.

Figure 10 shows the amino acid sequence of the hinge, CH2 and CH3 regions of human IgG γ 1.

Figure 11 shows the cDNA and amino acid sequence of Epo N47-Fc fusion polypeptide including the Epo signal sequence. The amino terminal Fc residue is fused to the arg-166 residue of Epo.

Detailed Description of the Invention

The invention provides for a method of raising and maintaining hematocrit comprising administering a therapeutically effective amount of a hyperglycosylated analog of erythropoietin in a pharmaceutical composition. The analog is administered less frequently than an equivalent molar amount of rHuEpo to obtain a comparable target hematocrit. The invention also provides for a method of raising and maintaining hematocrit comprising administering a hyperglycosylated analog in lower molar amounts than rHuEpo to obtain a comparable target hematocrit. The composition may be administered by intravenous, subcutaneous or intraperitoneal routes.

Surprisingly, it has been found that analog N47, a hyperglycosylated Epo analog described in WO94/09257, could achieve an increase in hematocrit

administered once a week that was comparable to that observed for rHuEpo given three times per week. Analog N47 has the following amino acid changes: ala to asn at 30; his to thr at 32; pro to val at 87; trp to asn at 88; and pro to thr at 90 which resulted in the addition of two N-linked carbohydrate chains at asparagine residues 30 and 88. The analog was expressed in Chinese hamster ovary (CHO) cells (as described in Example 1) and purified as described in Example 2 to give isoforms of 17 to 22 sialic acids. Analog N47 showed a greater serum half-life in rats and beagle dogs than rHuEpo when injected intravenously (Figures 4 and 5). When injected intraperitoneally three times per week, N47 induced increases in hematocrit of normal mice comparable to rHuEpo at lower concentrations (Figure 6). The potency of N47 was demonstrated to be about 3 to 4-fold higher than rHuEpo when administered three times per week (Figures 6 and 7). When given once per week, at similar doses, rHuEpo showed little stimulation of hematocrit in normal mice while N47 gave a marked increase (Figure 8). The potency of N47 was about 14-fold higher than rHuEpo for once per week dosing (Figure 7). Significantly, the hematocrit response for analog N47 given once per week is comparable to that for rHuEpo given three times per week. Even when administered once every other week, N47 still produced significant increases in the hematocrit of normal mice (Figure 9). Taken together, the data indicated that Epo hyperglycosylated analogs, and analog N47 in particular, can be used advantageously to raise hematocrit using less frequent dosing than for current treatment with rHuEpo.

It has also been shown that the results described above obtained in mice may be extrapolated to humans. Pharmacokinetic parameters for administration of rHuEpo and analog N47 to 11 Continuous Ambulatory

Peritoneal Dialysis (CAPD) patients demonstrate that analog N47 has a three-fold longer serum half-life than rHuEpo (Example 6 and Table 5). These results suggest that Epo hyperglycosylated analogs allow less frequent dosing than rHuEpo in humans.

As used herein, the term "hyperglycosylated Epo analog" refers to Epo comprising at least one additional glycosylation site with an additional carbohydrate chain added to the site. Glycosylation sites may be for N-linked or O-linked carbohydrate chains. New N-linked glycosylation sites are introduced by alterations in the DNA sequence to encode the consensus site for N-linked carbohydrate addition (the amino acids Asn-X-Ser/Thr) in the polypeptide chain, while new O-linked sites are introduced by alterations in the DNA sequence to encode a serine or a threonine residue. The analogs are constructed by mutagenesis techniques for introducing additions, deletions or substitutions of amino acid residues that increase or alter sites in the Epo polypeptide that are available for glycosylation. DNA encoding an Epo hyperglycosylated analog is transfected into a eucaryotic host cell and the expressed glycoprotein is analyzed for the presence of an additional carbohydrate chain.

Epo hyperglycosylated analogs have shown in vitro activity which was comparable to or even less than that determined for rHuEpo, suggesting that binding to the Epo receptor is not enhanced, and may in some cases be diminished, by addition of carbohydrate chains. However, hyperglycosylation can typically increase serum half-life and potentially lead to increased in vivo biological activity. One Epo analog having an additional N-linked carbohydrate chain at position 88 exhibited decreased affinity for receptor compared to rHuEpo (isoforms 9-14) or to a purified

isoform of rHuEpo having 14 sialic acids per molecule, yet demonstrated a longer circulating half-life and enhanced in vivo activity compared to either a mixture of Epo isoforms 9-14 or isolated Epo isoform 14.

5 The Epo hyperglycosylated analogs which may be administered according to the present invention will have at least one additional N-linked or O-linked carbohydrate chain. In one embodiment, the analogs will have two additional N-linked carbohydrate chains.
10 In other embodiments, the analogs will have three, four or more additional N-linked carbohydrate chains. As examples, the analogs of the invention will have at least one additional N-linked chain at one or more of amino acid residues 30, 51, 57, 69, 88, 89, 136 and 138
15 of the sequence of human Epo. In one embodiment, the analog has additional N-linked carbohydrate chains at residues 30 and 88 of human Epo. The numbering of amino acid residues of human Epo is as shown in Figure 1 and SEQ ID NO:1. Figure 1 shows a predicted mature
20 Epo polypeptide of 166 amino acids whereas recombinant produced Epo has 165 amino acids after removal of the C-terminal arginine residue. It is understood that rHuEpo and hyperglycosylated Epo analogs may have either 165 or 166 amino acids.

25 The analogs of the invention will have at least four N-linked carbohydrate chains. Of the four chains, three may be at the naturally occurring sites at positions 24, 38, and 83. However, it is contemplated that some analogs of the invention may
30 have alterations of one or more of the naturally-occurring glycosylation sites such that one or more of the sites are deleted and substituted with a new site. Such analogs are also provided by the invention. For example, any one of sites at positions 24, 38 and 83
35 may be deleted and substituted with a site at position

88. Optionally, the analogs may have an O-linked site at position 126.

The invention also provides for new Epo hyperglycosylated analogs having at least one additional carbohydrate chain. It has been found that an additional N-linked carbohydrate chain is added at any of positions 52, 53, 55, 86 and 114 which have been modified to be a glycosylation site. Specific embodiments include analogs N49 through N61 as described in Table 1. The new analogs will have at least one new N-linked glycosylation site at any of positions 52, 53, 55, 86 and 114 and may further comprise additional N-linked or O-linked carbohydrate chains at other sites. The analogs may have one, two, three or four additional carbohydrate chains, or more than four additional chains. In one preferred embodiment, the analogs will have three additional N-linked carbohydrate chains (six N-linked chains total). In another preferred embodiment, the analogs will have four additional N-linked chains (seven N-linked chains total). The analogs having three or four, or more than four, additional N-linked carbohydrate chains may have, but are not limited to, an additional chain at any of positions 52, 53, 55, 86 and 114.

Surprisingly, it has been found that a hyperglycosylated analog with three additional N-linked chains at positions 30, 53 and 88 (six N-linked chains total) has a greater in vivo activity than analog N47 with two additional chains (five total). The results are shown in Figure 3. It is clear that the in vivo activity of the analogs is directly dependent on the number of N-linked carbohydrate chains. These results may be extrapolated to the therapeutic setting wherein the analogs having more N-linked carbohydrate chains than N47 may be dosed even less frequently.

In addition, the invention provides for hyperglycosylated Epo analogs with three additional N-linked chains at positions 30, 55 and 88; 30, 55 and 114; and 30, 88 and 114. Epo analogs with four
5 additional N-linked chains or three additional N-linked chains and one additional O-linked chain at position 125 are also provided.

The analogs may be prepared by a variety of mutagenesis techniques available to one skilled in the
10 art, such as site-directed mutagenesis, PCR mutagenesis and cassette mutagenesis (Zoller et al. Meth. Enz. 100, 468-500 (1983); Higuchi, in PCR Protocols pp. 177-183 (Academic Press, 1990); Wells et al. Gene 34, 315-323 (1985)). Example 1 describes the use of PCR
15 mutagenesis techniques to construct new Epo hyperglycosylated analogs.

An Epo DNA sequence which has undergone mutagenesis is inserted into an expression vector using standard techniques with the vector being suitable for
20 maintenance in a mammalian host cell. The vector will typically contain the following elements: promoter and other "upstream" regulatory elements, origin of replication, ribosome binding site, transcription termination site, polylinker site, and selectable
25 marker that are compatible with use in a mammalian host cell. Vectors may also contain elements that allow propagation and maintenance in procaryotic host cells as well.

Suitable cells or cell lines include any from
30 mammalian sources, including human sources. Examples include COS-7 (ATCC accession no. CRL 1651), human 293, baby hamster kidney (BHK, ATCC accession no. CCL 10), Chinese hamster ovary cells (including dihydrofolate reductase (DHFR)-deficient cells, Urlab et al. Proc.
35 Natl. Acad. Sci. USA 77, 4216-4220 (1980)) Other suitable mammalian cell lines include, but are not

limited to, HeLa, mouse L-929 and 3T3. In a preferred embodiment, DHFR-deficient CHO cells are used.

5 Vectors comprising sequences encoding Epo hyperglycosylation analogs are introduced into host cells by standard transformation or transfection techniques. Culturing, amplifying and screening transformed or transfected host cells are accomplished using publicly available methods (Gething et al. *Nature* 293, 620-625 (1981); Kaufman et al. *Mol Cell. Biol.* 5, 10 1750-1759 (1985); U.S. Patent No. 4,419,446). Host cells harboring DNA sequences encoding Epo hyperglycosylated analogs are cultured under conditions that permit expression of the analogs. The analogs are recovered from the cell media and purified using 15 procedures essentially as described previously (WO94/09257) and those in Example 2. The purification procedures allow for the isolation of higher sialic acid containing Epo isoforms resulting from adding additional carbohydrate chains.

20 The Epo hyperglycosylated analogs may include, in addition to new glycosylation sites, additions, deletions or substitutions of amino acid residues which do not create new glycosylation sites and do not substantially alter the biological activity 25 of the hyperglycosylated analog. Those individual sites or regions of Epo which may be altered without affecting biological activity may be determined by examination of the structure of the Epo-Epo receptor complex as described in Syed et. al. *Nature* 395, 511 30 (1998). Examination of the structure of the Epo-Epo receptor complex reveals those residues which interact with, or are in close proximity to, the receptor binding site of Epo and which should be avoided when making alterations in the Epo amino acid sequence. 35 Alternatively, one may empirically determine those regions which would tolerate amino acid substitutions

by alanine scanning mutagenesis (Cunningham et al. Science 244, 1081-1085 (1989). In this method, selected amino acid residues are individually substituted with a neutral amino acid (e.g., alanine) 5 in order to determine the effects on biological activity.

It is generally recognized that conservative amino acid changes are least likely to perturb the structure and/or function of a polypeptide. 10 Accordingly, the invention encompasses one or more conservative amino acid changes within an Epo hyperglycosylated analog. Conservative amino acid changes generally involve substitution of one amino acid with another that is similar in structure and/or 15 function (e.g., amino acids with side chains similar in size, charge and shape). The nature of these changes are well known to one skilled in the art and are summarized in Table 1 below. Such conservative substitutions are shown under the heading of "Preferred 20 substitutions". Also contemplated are more substantial changes ("Exemplary substitutions") which may also be introduced. A skilled artisan will appreciate that initially the sites should be modified by substitution in a relatively conservative manner. If such 25 substitutions result in a retention in biological activity, then more substantial changes (Exemplary Substitutions) may be introduced and/or other additions/deletions may be made and the resulting products screened.

TABLE 1: Amino Acid Substitutions

<u>Original Residue</u>	<u>Preferred Substitutions</u>	<u>Exemplary Substitutions</u>
Ala (A)	Val	Val; Leu; Ile
Arg (R)	Lys	Lys; Gln; Asn
Asn (N)	Gln	Gln; His; Lys; Arg
Asp (D)	Glu	Glu
Cys (C)	Ser	Ser
Gln (Q)	Asn	Asn
Glu (E)	Asp	Asp
Gly (G)	Pro	Pro
His (H)	Arg	Asn; Gln; Lys; Arg
Ile (I)	Leu	Leu; Val; Met; Ala; Phe; norleucine
Leu (L)	Ile	norleucine; Ile; Val; Met; Ala; Phe
Lys (K)	Arg	Arg; Gln; Asn
Met (M)	Leu	Leu; Phe; Ile
Phe (F)	Leu	Leu; Val; Ile; Ala
Pro (P)	Gly	Gly
Ser (S)	Thr	Thr
Thr (T)	Ser	Ser
Trp (W)	Tyr	Tyr
Tyr (Y)	Phe	Trp; Phe; Thr; Ser
Val (V)	Leu	Ile; Leu; Met; Phe; Ala; norleucine

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Also provided by the invention are deletions or additions of amino acids in a hyperglycosylated Epo analog which do not substantially affect biological activity. Such additions and deletions may be at the
5 N-terminal or C-terminal of the polypeptide, or may be internal to it. In general, relatively small deletions or additions are less likely to affect structure and/or function of Epo or a hyperglycosylated analog. In one
10 embodiment, deletions or additions can be from 5-10 residues, alternatively from 2-5 amino acid residues, or from 1-2 residues.

The invention provides for fusion proteins comprising Epo hyperglycosylated analogs and compositions thereof. In one aspect, the invention
15 provides for fusion proteins of Epo hyperglycosylated analogs and an immunoglobulin heavy chain constant region. Fusions may be made at the amino terminus of an Epo hyperglycosylated analog, that is, the carboxy terminus of an immunoglobulin heavy chain constant
20 region is fused to the amino terminus of an Epo hyperglycosylated analog. Alternatively, it may be desirable to fuse the carboxy terminus of an Epo hyperglycosylated analog to the amino terminus of an immunoglobulin heavy chain constant region. In one
25 aspect of the invention, the immunoglobulin heavy chain constant region is an Fc region. Epo hyperglycosylated analogs, when part of a fusion polypeptide, may be 165 or 166 amino acids in length, or may have greater or fewer residues if amino acids are added or deleted. In
30 one embodiment, analog N47 is fused at its C-terminus to the N-terminus of an Fc region derived from human IgGyl. (See Example 2.) In the present example, analog N47 includes the arginine residue at position 166. However, it is contemplated that analog N47 as
35 well as other hyperglycosylated analogs of residues 1-

165 (lacking the C-terminal arginine residue) may also comprise the fusion polypeptides of the invention.

The term "Fc" refers to a molecule or
5 sequence comprising the sequence of a non-antigen-
binding portion of antibody, whether in monomeric or
multimeric form. The original immunoglobulin source of
an Fc is preferably of human origin and may be from any
isotype, e.g., IgG, IgA, IgM, IgE or IgD. One method
10 of preparation of an isolated Fc molecule involves
digestion of an antibody with papain to separate
antigen and non-antigen binding portions of the
antibody. Another method of preparation of an isolated
Fc molecules is production by recombinant DNA
15 expression followed by purification of the Fc molecules
so expressed. A full-length Fc consists of the
following Ig heavy chain regions: CH1, CH2 and CH3
wherein the CH1 and CH2 regions are typically connected
by a flexible hinge region. In one embodiment, an Fc
20 has the amino acid sequence of IgG1 such as that shown
in Figure 10. The terms "Fc protein", "Fc sequence",
"Fc molecule", "Fc region" and "Fc portion" are taken
to have the same meaning as "Fc".

The term "Fc fragment" when used in
25 association with Fc molecule, or fusion polypeptides
thereof, refers to a peptide or polypeptide that
comprises less than the full length amino acid sequence
of an Fc molecule. Such a fragment may arise, for
example, from a truncation at the amino terminus, a
30 truncation at the carboxy terminus, and/or an internal
deletion of a residue(s) from the amino acid sequence.
Fc fragments may result from alternative RNA splicing
or from *in vivo* protease activity.

The term "Fc variant" when used in
35 association with an Fc molecule, or with fusion
polypeptides thereof, refers to a polypeptide

comprising an amino acid sequence which contain one or more amino acid sequence substitutions, deletions, and/or additions as compared to native Fc amino acid sequences. Variants may be naturally occurring or
5 artificially constructed. Variants of the invention may be prepared from the corresponding nucleic acid molecules encoding said variants, which have a DNA sequence that varies accordingly from the DNA sequences for native Fc molecule.

10 The term "derivative" when used in association with an Fc molecule, or with fusion polypeptides thereof, refers to Fc variants or fragments thereof, that have been chemically modified, as for example, by covalent attachment of one or more
15 polymers, including, but limited to, water soluble polymers, N-linked or O-linked carbohydrates, sugars, phosphates, and/or other such molecules. The derivatives are modified in a manner that is different from native Fc, either in the type or location of the
20 molecules attached to the polypeptide. Derivatives further includes deletion of one or more chemical groups naturally attached to an Fc molecule.

The term "fusion" refers to joining of different peptide or protein segments by genetic or
25 chemical methods wherein the joined ends of the peptide or protein segments may be directly adjacent to each other or may be separated by linker or spacer moieties such as amino acid residues or other linking groups.

An Fc, or a variant, fragment or derivative
30 thereof, may be from an Ig class. In one embodiment, an Fc is from the IgG class, such as IgG1, IgG2, IgG3, and IgG4. In another embodiment, an Fc is from IgG1. An Fc may also comprise amino acid residues represented by a combination of any two or more of the Ig classes,
35 such as residues from IgG1 and IgG2, or from IgG1, IgG2 and IgG3, and so forth. In one embodiment, an Fc

region of an Epo hyperglycosylated analog fusion protein has the sequence as set forth in Figure 10 (SEQ ID NO:___) (see Ellison et al., Nucleic Acids Res. 10, 4071-4079 (1982)) starting at residue 6 (that is, residues 1-5 are deleted).

In addition to naturally occurring variations in Fc regions, Fc variants, fragments and derivatives may contain non-naturally occurring changes in Fc which are constructed by, for example, introducing substitutions, additions, insertions or deletions of residues or sequences in a native or naturally occurring Fc, or by modifying the Fc portion by chemical modification and the like. In general, Fc variants, fragments and derivatives are prepared such that the increased circulating half-life of Fc fusions to Epo glycosylation analogs is largely retained.

Also provided by the invention are Fc variants with conservative amino acid substitutions. Examples of conservative amino acid substitutions are set forth hereinabove, and are also exemplified by substitution of non-naturally occurring amino acid residues which are typically incorporated by chemical peptide synthesis rather than by synthesis in biological systems. These include peptidomimetics, and other reversed or inverted forms of amino acid moieties. Conservative modifications to the amino acid sequence of an Fc region (and the corresponding modifications to the encoding nucleotides) are expected to produce Fc molecules (and fusion proteins comprising Epo hyperglycosylated analogs and Fc regions) which have functional and chemical characteristics similar to those of unmodified Fc molecules and fusion proteins comprising unmodified Fc regions.

In addition to the substitutions set forth in Table I, any native residue in an Fc molecule (or in an Fc region of a fusion protein comprising an Epo

hyperglycosylated analog) may also be substituted with alanine, as has been previously described for "alanine scanning mutagenesis" (Cunningham et al. Science 244, 1081-1085 (1989)).

5 Substantial modifications in the functional and/or chemical characteristics of an Fc molecule (and in an Fc region of a fusion protein comprising an Epo hyperglycosylated analog) may be accomplished by selecting substitutions that differ significantly in
10 their effect on maintaining (a) the structure of the molecular backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally
15 occurring residues may be divided into groups based on common side chain properties:

- 1) hydrophobic: norleucine, Met, Ala, Val, Leu, Ile;
- 2) neutral hydrophilic: Cys, Ser, Thr;
- 20 3) acidic: Asp, Glu;
- 4) basic: Asn, Gln, His, Lys, Arg;
- 5) residues that influence chain orientation: Gly, Pro; and
- 6) aromatic: Trp, Tyr, Phe.

25 Non-conservative substitutions may involve the exchange of a member of one of these classes for a member from another class. Such substituted residues may be introduced into regions of an Fc molecule that are homologous with a non-human Fc molecule, or into
30 the non-homologous regions of the molecule.

Cysteine residues in Fc molecules can be deleted or replaced with other amino acids to prevent formation of disulfide crosslinks. In particular, a cysteine residue at position 5 of Figure 10 (SEQ. ID.
35 NO. __) may be substituted with one or more amino

acids, such as alanine or serine. Alternatively, the cysteine residue at position 5 could be deleted.

An Fc fragment may be prepared by deletion of one or more amino acids at any of positions 1, 2, 3, 4 and 5 as shown in Figure 10 (SEQ ID NO. ____). In one embodiment, the amino acid residues at positions 1-5 inclusive are deleted. Substitutions at these positions can also be made and are within the scope of this invention.

10 Fc variants may also be made which show reduced binding to Fc receptors which trigger effector functions such as antibody dependent cellular cytotoxicity (ADCC) and activation of complement (see for example Molec. Immunol. 29, 633-639, (1992)). Such
15 variants may include leucine at position 20 deleted or substituted with a glutamine residue, glutamate at position 103 deleted or substituted with an alanine residue, and lysines at positions 105 and 107 deleted or substituted with alanine residues (following the
20 numbering as set forth in Figure 1). One or more of such substitutions are contemplated.

In one embodiment, Fc variants will exhibit stronger binding to the FcRn receptor ("salvage receptor") and a longer circulating half-life compared
25 to native Fc such as that shown in Figure 1. Example of such variants include amino acid substitutions at one or more of residues 33, 35-42, 59, 72, 75, 77, 95-98, 101, 172-174, 215 and 220-223, wherein the substitution(s) confer tighter binding of an Fc variant
30 to the FcRn receptor. In another embodiment, Fc variants have one or more glycosylation sites removed. N-linked glycosylation sites may be removed by deletion or substitution of asparagine residues having attached carbohydrate chains.

35 Other Fc variants include one or more tyrosine residues replaced with, for example,

phenylalanine residues. In addition, other variant amino acid insertions, deletions and/or substitutions are also contemplated and are within the scope of the present invention. Examples include Fc variants disclosed in WO96/32478 and WO97/34630 hereby incorporated by reference. Furthermore, alterations may be in the form of altered amino acids, such as peptidomimetics or D-amino acids.

The Fc protein may be also linked to the Epo glycosylation analogs by "linker" moieties comprising chemical groups or amino acids of varying lengths. Such chemical linkers are well known in the art. Amino acid linker sequences can include but are not limited to:

- (a) ala-ala-ala;
- (b) ala-ala-ala-ala;
- (c) ala-ala-ala-ala-ala;
- (d) gly-gly;
- (e) gly-gly-gly;
- (f) gly-gly-gly-gly-gly;
- (g) gly-gly-gly-gly-gly-gly-gly;
- (h) gly-pro-gly;
- (i) gly-gly-pro-gly-gly; and
- (j) any combination of subparts (a) through (i).

While Fc molecules are preferred as components of fusion proteins with Epo glycosylation analogs, it is also contemplated that other amino acid sequences which bind to an FcRn receptor and confer increased in vivo half-life may also be used. Examples of such alternative molecules are described in U.S. Pat. No. 5,739,277, issued April 14, 1998 to Presta et al.

The term "molar amount" refers to an amount of a hyperglycosylated analog or rHuEpo which is based upon the molecular weight of the corresponding erythropoietin polypeptide without glycosylation.

5 Equivalent amounts of rHuEpo and analog refer to amounts which are equal when taking into account normal variations in procedure used to determine such amounts. It is necessary to determine equivalent amounts in this manner since the molecular weight of rHuEpo and analogs
10 will vary depending upon the number of carbohydrate chains. For rHuEpo, the molecular weight of erythropoietin polypeptide is calculated based upon amino acid residues 1-165 as shown in Figure 1 and SEQ ID NO: 1. For hyperglycosylated analogs, the molecular
15 weights are adjusted depending upon the amino acid changes in residues 1-165 of Figure 1 and SEQ ID NO: 1.

The dosing frequency for a hyperglycosylated analog will vary depending upon the condition being treated and the target hematocrit, but in general will
20 be less than three times per week. The dosing frequency will be about two times per week, about one time per week. The dosing frequency may also be less than about one time per week, for example about one time every two weeks (about one time per 14 days), one
25 time per month or one time every two months. It is understood that the dosing frequencies actually used may vary somewhat from the frequencies disclosed herein due to variations in responses by different individuals to the Epo analogs; the term "about" is intended to
30 reflect such variations.

As used herein, the term "therapeutically effective amount" refers to an amount of a hyperglycosylated analog (or a fusion protein comprising an Epo hyperglycosylated analog and an
35 immunoglobulin heavy chain constant region) which gives an increase in hematocrit to a target hematocrit, or to

a target hematocrit range that provides benefit to a patient or, alternatively, maintains a patient at a target hematocrit, or within a target hematocrit range. The amount will vary from one individual to another and will depend upon a number of factors, including the overall physical condition of the patient, severity and the underlying cause of anemia and ultimate target hematocrit for the individual patient. A target hematocrit is typically at least about 30%, or in a range of 30%-38%, preferably above 38% and more preferably 40%-45%. General guidelines relating to target hematocrit ranges for rHuEpo are also found in the EPOGEN® package insert dated 12/23/96 and are 30%-36%, or alternatively 32%-38% as stated therein. It is understood that such targets will vary from one individual to another such that physician discretion may be appropriate in determining an actual target hematocrit for any given patient. Nonetheless, determining a target hematocrit is well within the level of skill in the art.

A therapeutically effective amount of the present compositions may be readily ascertained by one skilled in the art. Example 6 sets forth a clinical protocol which has as one objective to determine a therapeutically effective amount of analog N47 in both once per week and three times per week dosing. A dose range for once per week administration is from about 0.075 to about 4.5 µg erythropoietin peptide per kg per dose. A dose range for three times per week administration is 0.025 to 1.5 µg erythropoietin peptide per kg per dose. This dose range may be employed with other Epo hyperglycosylated analogs, with any adjustments in the dosing range being routine to one skilled in the art.

A significant advantage to the present invention is the ability to correlate the extent of hyperglycosylation either with a dose amount or with a dosing interval that would allow one to "tailor" an Epo analog to a given dose or dosing schedule. Based upon the increasing in vivo activities of Epo analogs having one, two or three additional carbohydrate chains as shown in Figure 3, the treating physician can select an analog that is appropriate and convenient for the anemic condition being treated. For example, in patients who are acutely anemic and in need of a large effective dose, or in patients which require a longer-lasting treatment, administration of a hyperglycosylated analog with three or four or even more additional carbohydrate chains may be preferred. For other patients who experience less severe anemia or require treatment for a relatively short time, an analog with one or two additional carbohydrate chains may be preferred. The analogs of the present invention provide the physician with considerable flexibility in preventing and treating anemia that may result from a wide variety of underlying conditions.

The invention also provides for administration of a therapeutically effective amount of iron in order to maintain increased erythropoiesis during therapy. The amount to be given may be readily determined by one skilled in the art based upon therapy with rHuEpo.

The present invention may be used to stimulate red blood cell production and prevent and treat anemia. Among the conditions treatable by the present invention include anemia associated with a decline or loss of kidney function (chronic renal failure), anemia associated with myelosuppressive therapy, such as chemotherapeutic or anti-viral drugs (such as AZT), anemia associated with the progression

of non-myeloid cancers, anemia associated with viral infections (such as HIV), and anemia of chronic disease. Also treatable are conditions which may lead to anemia in an otherwise healthy individual, such as
5 an anticipated loss of blood during surgery. In general, any condition treatable with rHuEpo may also be treated with the Epo hyperglycosylated analogs of the invention.

The invention also provides for
10 pharmaceutical compositions comprising a therapeutically effective amount of an Epo hyperglycosylated analog, together with a pharmaceutically acceptable diluent, carrier, solubilizer, emulsifier, preservative and/or adjuvant.
15 The invention also provides for a pharmaceutical composition comprising a therapeutically effective amount of a fusion protein comprising an Epo hyperglycosylated analog and an immunoglobulin heavy chain constant region together with a pharmaceutically
20 acceptable diluent, carrier, solubilizer, emulsifier, preservative and/or adjuvant. The composition will be suitable for a dosing schedule of less than three times per week. The composition may be in a liquid or lyophilized form and comprises a diluent (Tris,
25 citrate, acetate or phosphate buffers) having various pH values and ionic strengths, solubilizer such as Tween or Polysorbate, carriers such as human serum albumin or gelatin, preservatives such as thimerosal, parabens, benzylalconium chloride or benzyl alcohol,
30 antioxidants such as ascorbic acid or sodium metabisulfite, and other components such as lysine or glycine. Selection of a particular composition will depend upon a number of factors, including the condition being treated, the route of administration
35 and the pharmacokinetic parameters desired. A more extensive survey of components suitable for

pharmaceutical compositions is found in Remington's
Pharmaceutical Sciences, 18th ed. A.R. Gennaro, ed.
Mack, Easton, PA (1980). In a preferred embodiment,
the Epo glycosylation analogs of the invention are
5 formulated in liquid form in an isotonic sodium
chloride/sodium citrate buffered solution containing
human albumin, and optionally containing benzyl alcohol
as a preservative. The compositions preferably contain
analogs having one, two, three, four, or more
10 additional carbohydrate chains.

Compositions of the invention are preferably
administered by injection, either subcutaneous or
intravenous. The route of administration eventually
chosen will depend upon a number of factors and may be
15 ascertained by one skilled in the art.

The following examples are offered to more
fully illustrate the invention, but are not to be
construed as limiting the scope thereof.

20

EXAMPLE 1

Construction of Hyperglycosylated Epo Analogs

25 Construction of cDNAs encoding Hyperglycosylated Epo
Analogues

Epo analogs were made by in vitro mutagenesis
using several different methods. Analogs N49 and N50
were constructed as described in WO94/09257. Analogs
were also constructed by variations of overlap PCR
30 (polymerase chain reaction) methods. The basic
procedure included two successive steps. In the first
step, two reactions (PCR1 and PCR2) were performed on
Epo or Epo analog template DNA using a total of four
oligonucleotides: a 5' (forward) primer, a reverse
35 mutagenic primer, a forward mutagenic primer (usually
complementary to the reverse mutagenic primer) and a 3'

(reverse) primer. The mutagenic primers contained the desired nucleotide changes as well as 6-14 exact match nucleotides on each side of these changes. PCR1 used the 5' (forward) primer and the reverse mutagenic primer. PCR2 used the 3' (reverse) primer and the forward mutagenic primer. The amplified DNA fragments were separated by agarose gel electrophoresis. Small pieces of agarose containing DNA fragments of the correct size were excised from the gel. The DNA fragments from PCR1 and PCR2 were combined together and a third PCR reaction was performed using only the 5' forward and 3' reverse primers. Thus, a full length DNA segment containing the desired mutations was amplified. In several cases, two or three mutations were combined by introducing a new substitution into DNA already containing a change, using the same PCR process. To construct these multiple glycosylation site analogs, single double or triple site analogs (produced as described above) were used as PCR template, and an additional glycosylation site was introduced by site directed mutagenesis with the appropriate primers.

The Epo analogs N51, N52 and N53 were constructed by the overlap PCR (polymerase chain reaction) method 1. One additional N-glycosylation site was introduced in each case. N56 added a glycosylation site (N114 T116) to native sequence HuEpo by using pDSR α 2 Epo as PCR template, N51 added an O-linked glycosylation site (Thr125) to N47 Epo by using pDSR α 2 Epo N47 template (Asn30, Thr32, Val87, Asn88, Thr90) and analog N59 added a glycosylation site (Asn53) to analog N47 using pDSR α 2 EpoN47 template.

Polymerase chain reactions for method 1 were performed using a protocol adapted from Cheng et. al.,

(Proc. Natl. Acad. Sci. USA 91, 5695 (1994)). The 3' (reverse) primer contained sequences that introduced a stop codon followed by a Xba I restriction site:

ATCTAGAAGTTGCTCTCTGGACAGTTCCT (SEQ ID NO: 2).

- 5 The 5' forward reaction primer:

GAAGCTTGCGCCACCATGGGGGTGCACGAATG (SEQ ID NO: 3)

had an Hind III restriction site followed by a Kozak sequence upstream of the Epo initiator codon (ATG).

The typical PCR reaction mix contained: 4 µl each of

- 10 forward and reverse primers (5 pmol/µl), 1 µl template (25 ng), 10 µl of 5X LP buffer (100 mM Tricine pH 8.7/25% glycerol/425 mM KOAc), 10 µl dNTP stock (1 mM each of dATP, dTTP, dCTP, dGTP), 0.8 µl rtTh polymerase (Perkin Elmer; 2.5 U/µl), and 2 µl Vent polymerase
- 15 (NEB; 0.01 U/µl after 1:100 fresh dilution in 1X LP buffer). H₂O was added to bring the final volume to 50 µl. All the components were added together in the order shown and the PCR was started when the temperature during the first cycle was above 60°C by
- 20 adding 1 µl of 50 mM MgOAc. Typical reaction conditions were: 2 cycles of 94°C, 10 sec/50°C, 1 min./ 68°C, 5 min. followed by 25 cycles of 94°C, 10 sec/55°C, 1 min./ 68°C, 5 min. The amplified fragments were separated by agarose gel electrophoresis and the
- 25 correct sized DNA fragment was purified using a Geneclean™ kit and procedures supplied by the manufacturer (Bio 101, Inc.). The purified DNA was digested with Hind III and Xba I, then it was purified again using the Geneclean™ kit. The fragment was then
- 30 ligated into Hind III and Xba I cut pDSR02 vector. Ligated DNA was precipitated with 2 volumes of ethanol

in 0.3M NaOAc pH 5.2 in the presence of carrier tRNA and transformed into *E. coli*. Epo analogs were screened by restriction digest on mini DNA preps. Plasmids from positive clones were then prepared and the insert was
5 sequenced to confirm the presence of the desired mutations and to ensure that no additional amino acid changes were introduced.

10 Analog N54 to N61 were constructed using overlap PCR strategy method 2. The 3' (reverse) primer contained sequences that introduced a stop codon followed by a XbaI restriction site:

GATCCTCTAGAGTTGCTCTCTGGACAG (SEQ ID NO: 4).

The 5' forward reaction primer:

CAACAAGCTTGCGCCGCCATGGGGG (SEQ ID NO: 5)

15 had a HindIII restriction site followed by a Kozak sequence upstream of the Epo initiator codon (ATG). A high fidelity PCR strategy was performed using Perkin Elmer ULTma DNA Polymerase and accompanying reagents; 10 µl 10X PCR buffer, 3 µl 1 mM dNTPs, 5 pmol of each
20 primer, and water in a final volume of 100 µl. 0.5 units of ULTma polymerase was added after the PCR mixture reached 94°C. PCR reactions were then carried out for 5 cycles at 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 90 seconds. A subsequent 25
25 cycles were performed at 94°C for 30 seconds, and 72°C for 90 seconds. Product bands of the correct sizes were excised from an agarose gel following electrophoresis.

30 The resulting PCR products for each analog were cleaned using the Qiagen gel extraction kit. The purified DNA was digested in a 100 µl restriction digest with HindIII and XbaI restriction enzymes

(Boehringer Mannheim) at 37° C for 1 hour. The digests were again gel purified and the digested fragment was then ligated into HindIII and XbaI digested pDSRα2 vector.

- 5 Ligated DNA was precipitated with 2 volumes of ethanol in 0.3M NaOAc pH 5.2 in the presence of carrier tRNA and transformed into E. coli. Epo hyperglycosylated analogs were initially screened by colony PCR to identify clones containing the correctly
10 sized and type of DNA insert. With this procedure, cells containing plasmids were placed into PCR tubes in the presence of Epo forward and reverse primers. The mixture was then subjected to PCR using the reaction conditions described above. Plasmids from positive
15 clones were then prepared and the Epo analog insert was sequenced to confirm the presence of the desired mutations and to ensure that no additional amino acid changes were introduced.

TABLE 1
ERYTHROPOIETIN ANALOGS HAVING SITES
FOR N-LINKED CARBOHYDRATE CHAINS

Analog	Amino Acid Substitution	Sequence Changes
N49	Lys, Met → Asn52, Thr54	AAG, ATG → AAT, ACC
N50	Arg, Glu → Asn53, Thr55	AGG, GAG → AAT, ACG
N51	Ala, His, Pro, Trp, Pro, Ala → N30, T32, V87, N88, T90 T125	GCT, CAC, CCG, TGG, CCC, GCC → AAT, ACG, GTG, AAT, ACC, ACC
N52	Ala, Lys → Asn114, Thr116 →	GCC, AAG → AAC, ACG
N53	Ala, His, Arg, Glu Pro, Trp, Pro → N30, T32, N53, T55, V87, N88, T90	GCT, CAC, AGG, GAG, CCG, TGG, CCC → AAT, ACG, AAT, ACG, GTG, AAT, ACC
N54	Glu, Gly → Asn55, Thr57	GAG, GGG → AAT, ACT
N55	Gln, Pro, Trp → Asn86, Val87, Thr88	CAG, CCG, TGG → ACC, GTG, ACG
N56	Pro, Trp, Pro → Ala87, Asn88, Thr90	CCG, TGG, CCC → GCG, AAT, ACC
N57	Pro, Trp, Pro → Val87, Asn88, Ser90	CCG, TGG, CCC → GTG, AAT, ACG
N58	Pro, Trp, Glu, Pro → Val87, Asn88, Gly89, Thr90	CCG, TGG, GAG, CCC → GTG, AAT, GGG, ACC
N59	Ala, His, Arg, Glu → Asn30, Thr32, Asn53, Asn55	GCT, CAC AGG GAG → AAT, ACG, AAT, ACG
N60	Ala, His, Ala, Lys → Asn30, Thr32, Asn114, Thr116	GCT, CAC, GCC, AAG → AAT, ACG, AAC, ACG
N61	A, H, R, E, P, W, P, A, K → 10, T32, N53, T55, V87, N88, T90, N114, T115	GCT, CAC, ACG, GAG, CCG, TGG, CCC, GCC, AAG → AAT, ACG, AAT, A CG, GTG, AAT, ACC, AAC, ACG

Analysis of carbohydrate addition

The constructs for the hyperglycosylated Epo
5 analogs which were inserted into the expression vector
pDSR02 were transfected into COS cells. Supernatants
from the transfected COS cells were analyzed by western
blot to determine whether the expressed and secreted
Epo analog contained additional carbohydrate. Samples
10 were loaded directly into wells of SDS-PAGE gels then
analyzed by immunoblot using the monoclonal antibody,
9G8A (Elliott et al (1996) Blood 87:p2714). Mobilities
of analog samples were compared to that of samples

containing rHuEpo. Figure 1 shows decreased mobility of analogs N53 and N61 compared to analogs N4 (four carbohydrate chains) and N47 (five carbohydrate chains). The mobility is consistent with the presence of six carbohydrate chains for analog N53 and seven carbohydrate chains for analog N61. Data for all hyperglycosylated analogs are shown in Table 2.

In vitro bioassays

Media conditioned by COS or CHO cells expressing rHuEpo or analogs were assayed for stimulation of 3H-thymidine uptake by UT7-Epo cells (Komatsu et al., Blood 82,456). UT7-Epo cells are responsive to Epo and express human Epo receptors on their cell surface. UT7-Epo cells were grown in Growth medium (1X Iscove's Modified Dulbecco's Medium with L-glutamine, 25 mM HEPES buffer, and 3024 mg/L sodium bicarbonate, but without either alpha-thioglycerol or beta-mercaptoethanol (GIBCO)/ 10% v/v Fetal Bovine Serum/ 1% v/v L-glutamine-Penicillin-Streptomycin solution (Irvine Scientific)/ 1 Unit/mL rHuEpo) to approximately 3×10^5 cells/mL. Cells were collected by centrifugation (approx. 500xG) washed twice with phosphate buffered saline and resuspended at 5×10^4 cells/mL in Assay medium (1x RPMI Medium 1640 without L-glutamine (Gibco)/1% L-glutamine/4% fetal bovine serum). Test samples or Epo standard (rHuEpo), 100 uL diluted in assay medium at least 5-fold, were added to wells in a 96 well microtiter plate. 50 uL of suspended cells were then added (5000 cells/well) and plates were incubated in a humidified incubator at 37°C and 5% CO₂. After 72 hours, 50 uL methyl-³H-Thymidine (1 mCi/mL; 20 Ci/mMole) diluted 1:100 in assay medium was added. Cells were incubated for an additional 4 hours at 37°C

and 5% CO₂. Labeled cells were harvested onto glass fiber filtermats, washed with deionized water followed by 2-propanol, dried and counted. Activity was determined by comparing the response determined for each analog to that of the rHuEpo standard. The specific biological activity was then determined by dividing in vitro activity by the concentration of each analog as determined by immunoassay (Elliott et al (1996) Blood 87:p2714). The results are shown in Table 2.

TABLE 2

ANALOG	Number of N-linked Carbohydrate Chains	In Vitro Activity **
rHuEpo	3	+++
N49	4	+++
N50	4	+++
N51	5*	+++
N52	3 - 4	+++
N53	6	++
N54	4	NT
N55	4	+++
N56	4	+++
N57	3 - 4	+++
N58	4	+++
N59	5	++
N60	4 - 5	+++
N61	6 - 7	NT

* contains 1-2 O-linked chains
 ** In vitro activity is relative to rHuEpo activity
 +++ activity equivalent to rHuEpo
 ++ activity is 25-75% of rHuEpo
 NT Not Tested

Epo analogs N62-N69 were made by overlap PCR (polymerase chain reaction) methods. The basic procedure included two successive steps. In the first step, two reactions (PCR1 and PCR2) were performed on Epo or Epo analog template DNA using a total of four oligonucleotides: a 5' (forward) primer, a reverse

mutagenic primer, a forward mutagenic primer complementary to the reverse mutagenic primer and a 3' (reverse) primer. The mutagenic primers contained the desired nucleotide changes as well as 6-14 exact match nucleotides on each side of these changes. PCR1 used the 5' (forward) primer and the reverse mutagenic primer. PCR2 used the 3' (reverse) primer and the forward mutagenic primer. The amplified DNA fragments were separated by agarose gel electrophoresis and DNA fragments of the correct size were excised and eluted from the gel. The DNA fragments from PCR1 and PCR2 were combined together and a third PCR reaction was performed using only the 5' forward and 3' reverse primers. For some analogs, three PCR reactions were required to generate the desired sequence. These were carried out as above, with a second pair of mutagenic primers being used to generate the third product. Again, the amplified DNA fragments were gel purified and combined in a final reaction containing only the 5' forward and 3' reverse primers. In each case, a full length DNA segment containing the desired mutations was amplified.

N62 added two glycosylation sites (N30 T32 N55 T57) to native sequence HuEpo by using pDSR α 2 Epo N4 (N30 T32) as PCR template. N63 added three glycosylation sites (N30 T32 N55 T57 V87 N88 T90) to native sequence HuEpo by using pDSR α 2 Epo N4 (N30 T32) and pDSR α 2 Epo N47 (N30 T32 N55 T57) as PCR templates. N64 added three glycosylation sites (N30 T32 N55 T57 N114 T116) to native sequence HuEpo by using pDSR α 2 Epo N4 (N30 T32) and pDSR α 2 Epo N60 as PCR templates. N65 added three glycosylation sites (N30 T32 V87 N88 T90 N114 T116) to native sequence HuEpo by using pDSR α 2 Epo N4 (N30 T32) and pDSR α 2 Epo N60 as PCR templates. N66

added four glycosylation sites (N30 T32 N55 T57 V87 N88 T90 N114 T116) to native sequence HuEpo by using pDSR α 2 Epo N4 (N30 T32) and pDSR α 2 Epo N60 as PCR templates. N67 added an O-linked glycosylation site (P124 T125 T 126)to N64 Epo. N68 added an O-linked glycosylation site (P124 T125 T 126)to N65 Epo. N69 added an O-linked glycosylation site (P124 T125 T 126)to N66 Epo.

For each analog, the same outside primers were used. The 3' (reverse) primer contained sequences that introduced a stop codon followed by a Sal I restriction site:

AGGTGGACAGTCGACATTATCTGTCCCCTGTC (SEQ ID NO:___).

The 5' forward reaction primer:

AACAAGCTTCTAGACCACCATGGGGGTG (SEQ ID NO: __)

had a Hind III restriction site followed by a Kozak sequence upstream of the Epo initiator codon (ATG).

Mutagenic primers were as follows:

N30 T32 mutagenic forward primer

ACG ACG GGC TGT AAT GAA ACG TGC AGC TTG (SEQ ID NO:___)

N30 T32 mutagenic reverse primer

CAA GCT GCA CGT TTC ATT ACA GCC CGT CGT G (SEQ ID NO:___)

N55 T57 mutagenic forward primer

GCC TGG AAG AGG ATG AAT GTC ACGCAG CAG GCC GTA GAA (SEQ ID NO: __)

N55 T57 mutagenic reverse primer

TTC TAC GGC CTG CTG CGT GAC ATTCAT CCT CTT CCA GGC A (SEQ ID NO: __)

V87 N88 T90 mutagenic forward primer

TCT TCC CAG GTG AAT GAG ACC CTG CAG CTG (SEQ ID NO: __)

V87 N88 T90 mutagenic reverse primer

CAG CTG CAG GGT CTC ATT CAC CTG GGA AGA GTT G (SEQ ID NO: __)

P124 T125 T126 mutagenic forward primer

5 CCA GAT CCG ACC ACA GCT GCT CCA (SEQ ID NO:)

P124 T125 T126 mutagenic reverse primer

TGG AGC AGC TGT GGT CGG ATC TGG A (SEQ ID NO:)

10 The N114 T116 changes were introduced using a template containing the appropriate mutations, so no mutagenic primers were required to generate this site.

The typical PCR1 reaction mix contained: 2.5 µl each of forward and mutagenic reverse primers (10 pmol/µl), 1 µl template (25 ng), 10 µl of 10X Taq Extend buffer (Stratagene), 2 µl dNTP stock (10 mM each of dATP, dTTP, dCTP, dGTP), 0.5 µl Taq polymerase (BMB), and 0.5 µl Taq Extend (Stratagene). H₂O was added to bring the final volume to 100 µl. Typical reaction conditions were: 1 cycle of 94°C, 5 min./55°C, 1 min./ 68°C, 1 min. followed by 25 cycles of 94°C, 1 min./55°C, 1 min./ 68°C, 1 min. The typical PCR2 reaction was identical to that described for PCR 1, except that the reverse and mutagenic forward primers were used. Where a third initial reaction was required, the reaction contained mutagenic forward and mutagenic reverse primers. The amplified fragments were separated by agarose gel electrophoresis and the correct sized DNA fragment was purified using a Gel Extraction kit and procedures supplied by the manufacturer (Qiagen). Complementary fragments were then combined in a third PCR reaction using only the outside forward and reverse primers. The amplified fragments were separated by

agarose gel electrophoresis and purified from the gel as described above. The purified DNA was digested with Hind III and Sal I, then it was again gel purified. The fragment was then ligated into Hind III and Sal I cut pDSR019 vector. Ligated DNA transformed by electroporation into *E. coli*. Epo hyperglycosylated analogs were initially screened by colony PCR to identify clones containing the correctly sized DNA insert. Plasmid DNA from selected clones was then prepared and the insert was sequenced to confirm the presence of the desired mutations and to ensure that no additional amino acid changes were introduced.

TABLE 3
ERYTHROPOIETIN ANALOGS HAVING SITES
FOR N-LINKED CARBOHYDRATE CHAINS

Analog	Amino Acid Substitution	Sequence Changes
N62	Ala, His, Glu, Gly → Asn30, Thr32, Asn55 Thr57	GCT,CAC,GAG, GGG→ AAT,ACG,AAT,ACT
N63	A,H,E,G,P,W,P→ N30, T32, N55,T57,V87, N88,T90	GCT,CAC,GAG, GGG, CCG, TGG, CCC→ AAT,ACG,AAT,ACT GTG, AAT, ACC
N64	A,H,E,G,A,K→ N30, T32, N55,T57,N114 T116	GCT,CAC,GAG,GGG,GCC,AAG→ AAT,ACG,AAT,ACT,AAC,ACG
N65	A,H,P,W,P, A, K→ N30, T32, V87, N88,T90, N114, T116	GCT,CAC, CCG, TGG, CCC, GCC, AAG→ AAT,ACG,GTG, AAT, ACC, AAC, ACG
N66	A,H,E,G,P,W,P, A, K→ N30,T32, N55,T57,V87, N88,T90, N114, T116	GCT,CAC,GAG, GGG, CCG, TGG, CCC, GCC, AAG→ AAT,ACG,AAT,,ACT GTG, AAT, ACC, AAC, ACG
N67	A,H,E,G,P,W,P, A, A, S→ N30, T32, N55,T57,V87, N88,T90, P124, T125, T126	GCT,CAC,GAG, GGG, CCG, TGG, CCC,GCG,GCC,TCA→ AAT,ACG,AAT,ACT GTG,AAT,ACC , CCG ACC ACA
N68	A,H,E,G,A,K, A, A, S→ N30, T32, N55,T57,N114 T116, P124, T125, T126	GCT,CAC,GAG,GGG,GCC,AAG, GCG,GCC,TCA → AAT,ACG,AAT,ACT,AAC,ACG, CCG, ACC,ACA
N69	A,H,E,G,P,W,P, A, K, A, A, S→ N30,T32, N55,T57,V87, N88,T90, N114, T116, P124, T125, T126	GCT,CAC,GAG, GGG, CCG, TGG, CCC, GCC,AAG, GCG,GCC,TCA → AAT,ACG,AAT,ACT GTG, AAT, ACC, AAC, ACG, CCG, ACC ,ACA

N70

Ala, His, Pro, Trp, Pro →
N30, T32, V87, N88, T90, IgG1 fusionGCT, CAC, CCG, TGG, CCC → AAT, ACG,
GTG, AAT, ACC

Construction of cDNA encoding Hyperglycosylated Epo
Analog Fusion Polypeptide

5 Epo analog N70 was also made by overlap PCR.
Plasmid DSR02 containing the cDNA sequence encoding
analog N47 (N30 T32 V87 N88 T90) and plasmid pAMG21
(ATCC accession no. 98113) containing cDNA encoding an
Fc region were used as templates for the polymerase
10 chain reactions. The Fc portion of human immunoglobulin
IgG1 heavy chain from residue 104 of the hinge
domain(Asp-104) to the carboxyl terminus (Ellison et
al., *supra*, see also Figure 10 starting at aspartic
acid residue at position 6), was generated by PCR
15 amplification of a human spleen cDNA library
(Clontech). Overlapping PCR products were generated in
two reactions using the following oligonucleotide
primers

5' forward reaction primer 2343-85 (Epo specific):

20 AAC AAG CTT CTA GAC CAC CAT GGG GGT G (SEQ ID NO: __)

3' reverse reaction primer 2343-87 (homology to both
Epo and Fc):

AGG TGG ACA TGT GTG AGT TTT GTC TCT GTC CCC TCT CCT GCA
GGC CTC C (SEQ ID NO: __)

25 5' forward reaction primer 2343-86 (homology to both
Epo and Fc):

GAG GCC TGC AGG ACA GGG GAC AGA GAC AAA ACT CAC ACA TGT
CCA CCT (SEQ ID NO: __)

30

3' reverse reaction primer 2343-88 (specific to Fc):

TGG ACA GTC GAC ATT ATT TAC CCG GAG ACA GGG AGA GGC TCT
TCT GC (SEQ ID NO: __)

PCR1 contained 2.5 μ l each of forward (2343-85) and reverse (2343-87) primers (10 pmol/ μ l), while PCR2 contained 2.5 μ l each of forward (2343-86) and reverse (2343-88) primers (10 pmol/ μ l). Conditions were as described above. The resulting amplified products contained a region of overlap (48 nucleotides) encoding the last 8 amino acids of Epo and the first 8 amino acids of Fc. The complementary fragments were gel purified and combined in a third PCR reaction using only the outside forward and reverse primers. The amplified fragment was separated by agarose gel electrophoresis and purified from the gel as described above. The purified DNA was digested with Hind III and Sal I, then it was again gel purified. The fragment was then ligated into Hind III and Sal I cut pDSR α 19 vector. Ligated DNA transformed by electroporation into E. coli. Transformants were initially screened by colony PCR to identify clones containing the correctly sized DNA insert. Plasmid DNA from selected clones was then prepared and the insert was sequenced to confirm the sequence of the fusion protein and to ensure that no additional amino acid changes were introduced.

25 Analysis of carbohydrate addition

The constructs for the hyperglycosylated Epo analogs N62 to N69 and fusion protein (analog N70) were inserted into the expression vector pDSR α 19 and transfected into CHO cells. Supernatants from the transfected CHO cells are analyzed by western blot to determine whether the expressed and secreted Epo analogs contained additional carbohydrate using procedures described above for analogs N49 to N62.

In vitro bioassays

In vitro assays for analogs N62 to N70 expressed in CHO transfected cells are performed as described above for analogs N49 to N61.

EXAMPLE 2

Preparation of Recombinant Human Erythropoietin and Hyperglycosylated Erythropoietin Analogs

10

Recombinant human erythropoietin (rHuEpo) used for the experiments described herein was expressed by Chinese hamster ovary (CHO) cells transfected with a recombinant plasmid carrying the human erythropoietin gene. The recombinant product was recovered from the conditioned medium and purified essentially as described by Lai et al. supra. The resulting rHuEpo preparation has predominantly isoforms of 9 to 14 sialic acids as determined by isoelectric focusing.

20

Recombinant hyperglycosylated erythropoietin analogs were expressed in CHO cells transfected with a recombinant plasmid carrying the Epo analog gene as described W091/05867 and W094/09257 hereby incorporated by reference. The hyperglycosylated analogs were purified from culture supernatants as described below.

25

Concentration and diafiltration of conditioned media

Conditioned medium (serum free) from three successive harvests (5-8 days each) of the transfected CHO cell line was collected, filtered through a 0.45 μ m filter, concentrated about thirty fold, and diafiltered into 10 mM Tris, 20 μ M CuSO₄, pH 7.0 using a tangential-flow ultrafiltration system (Millipore) with a 10,000 molecular weight cutoff membrane. The dialfiltered media (DFM) was filtered (0.45 μ m) a

35

second time and stored at -20°C until used for purification.

Purification

- 5 All procedures were carried out at 2 to 8°C.

Anion - Exchange Chromatography (10)

The clarified DFM was applied to a Q-Sepharose Fast Flow column (Pharmacia, 6 cm x 18 cm) equilibrated in 10 mM bis Tris propane (BTP), pH 7.0 and washed with two column volumes of 10 mM BTP to elute all non-binding species. The following gradients were run depending upon whether the hyperglycosylated analog had four, five or six N-linked carbohydrate chains. All buffers used at this stage contain 1 mM glycine, 20 μ M CuSO₄, 6 M urea, 5 μ g/mL leupeptin and 1 μ g/mL pepstatin. For analogs with four N-linked carbohydrate chains, the gradient was 10 mM acetic acid, 0.1 mM NaCl to 500 mM acetic acid, 5 mM NaCl over 49 column volumes with a two column volume hold at high salt conditions. For analogs with five N-linked carbohydrate chains, the gradient was 0.7 M acetic acid, 7 mM NaCl to 1.0 M acetic acid, 12 mM NaCl over 30 column volumes with a two column volume hold at high salt conditions. For analogs with six N-linked carbohydrate chains, the gradient was 1.0 M acetic acid, 10 mM NaCl to 1.5 M acetic acid, 20 mM NaCl over 50 column volumes with a two column volume hold at high salt conditions. Following the gradient, the column was washed with two column volumes of 10 mM BTP, pH 7.0 and the high isoform fraction was eluted with 0.6 M NaCl, 100 mM BTP, pH 7.0.

Reversed Phase Chromatography (C4)

The high salt strip from the Q-Sepharose column (1Q) was applied to a Vydac C4 reversed phase column (30 μ particles, 4 cm x 18 cm) equilibrated in 20% ethanol, 10 mM BTP, pH 7.0 and eluted from the column with a thirty column volume gradient to 94% ethanol buffered in 10 mM BTP, pH 7.0. The pooled product peak, eluting in approximately 60% ethanol, was diluted with four volumes of 10 mM BTP, pH 7.0 to minimize possibility of aggregation in the presence of ethanol.

Anion - Exchange Chromatography (2Q)

The diluted eluate from the reversed phase column was applied to a second Q-Sepharose Fast Flow (Pharmacia, 3 cm x 9 cm) column equilibrated with 10 mM BTP, pH 7.0. The column was washed with equilibration buffer, and the hyperglycosylated Epo analog was eluted with 0.6 M sodium chloride, 20 mM sodium citrate, pH 6.0.

The purified protein was exchanged into 20 mM NaPO_4 , pH 6.0, 140 mM NaCl via centricon (10,000 molecular weight cutoff), followed by passage through a 0.2 μm filter and storage at 2-8°C.

EXAMPLE 3

In Vivo Bioactivity of rHuEpo and rHuEpo Analogs
Containing four, five and six N-linked
Carbohydrate Chains

The in vivo activity of Epo analogs containing four, five and six N-linked carbohydrate chains was compared with that of rHuEpo in the exhypoxic polycythemic mouse bioassay. This assay

quantifies the incorporation of ^{59}Fe into newly synthesized red blood cells as a measure of the increase in erythropoiesis in mice in response to an exogenously-administered test sample. The assay, performed as described below, is a modification of the method of Cotes and Bangham (Nature 191, 1065 (1961)).

In this assay, female BDF₁ mice are first preconditioned by exposure to low oxygen conditions in a hypobaric chamber (0.4 -0.5 atm.) for approximately 18 hours a day for 14 days. To compensate for the low oxygen conditions the mice respond by stimulating erythropoiesis to increase the number of red blood cells and thereby the relative oxygen-carrying capacity. After completion of the final hypobaric exposure, the mice are allowed to remain at ambient pressure for approximately 72 hours prior to the administration of test samples by intraperitoneal injection. At ambient pressure the mice are relatively polycythemic and respond by decreasing endogenous erythropoietin production and the rate of erythropoiesis. Five days after sample administration, 0.2 -0.3 μCi of $^{59}\text{FeCl}_3$ in 0.2 mL is injected intravenously into the tail vein. Forty-eight hours later, the animals are sacrificed and the increase in erythropoiesis produced by the test samples is determined by measuring the amount of ^{59}Fe incorporated in a 0.5 mL sample of whole blood.

An example of the results obtained when rHuEpo and five different Epo analogs, containing four, five or six N-linked carbohydrate chains were tested in this assay is shown in Figure 3. Each sample was assayed at six or seven different dilutions within an appropriate concentration range. All samples were diluted in phosphate-buffered saline containing 0.5% bovine serum albumin, and 0.4 mL of each dilution was

administered to five preconditioned mice. Forty-eight hours after the administration of ^{59}Fe , the amount incorporated in 0.5 mL of blood was measured by gamma counting. The results for each of the samples are plotted as the percent ^{59}Fe incorporated versus the log of the administered dose.

As shown in Figure 3, all five of the hyperglycosylated Epo analogs tested in this assay were more potent than rHuEpo. In addition, the potency of each analog was directly dependent on the number of N-linked carbohydrate chains, with those analogs having an increased number of carbohydrate chains having the greater activity. Thus, analog N53, which contains six N-linked carbohydrate chains, was the most potent analog. Analog N47, which contains five N-linked carbohydrate chains, was in turn, more potent than those analogs containing four N-linked chains. The potencies of the three analogs containing four N-linked carbohydrate chains (N4, N18 and N50) were approximately equal to each other and greater than that of rHuEpo.

In this experiment, the doses of rHuEpo and analogs containing four, five or six N-linked carbohydrate chains required to produce 40% ^{59}Fe incorporation were 10,700 ng, 640 ng, 140 ng and 38 ng, respectively. Based on the amount of material required to produce this level of erythropoiesis, Epo analogs containing four, five, or six N-linked carbohydrate chains are 17-fold, 77-fold and 280-fold more potent than rHuEpo.

EXAMPLE 4

IV Pharmacokinetics of rHuEpo and Epo analog
N47 in Rats and Beagle Dogs

5 Two separate studies in rats and dogs were performed to compare the pharmacokinetic parameters of Epo N47 analog and rHuEpo.

10 In the rat studies, 1 μ Ci ($\sim 0.1 \mu$ g of peptide/kg) of either 125 I-Epo N47 analog, or 125 I-recombinant human erythropoietin (Amersham) was injected intravenously into a surgically implanted carotid cannula in normal male Sprague-Dawley rats weighing between 314-363 g. At various time points after administration, 0.3mL of blood was collected and serum was prepared by centrifugation. The level of 125 I-rHuEpo or 125 I Epo N47 analog in 0.1mL of each serum sample was then determined following an overnight 4°C incubation with 90% ethanol. The ethanol-precipitated protein in each serum sample was collected by centrifugation and the radioactivity was counted in a gamma counter. The resulting serum concentration vs time pharmacokinetic curves are shown in Figure 4. Each point represents a group mean of five rats in the N47 analog group and six rats in the rHuEpo group. The pharmacokinetic parameters were determined for each rat using PCNONLIN 4.0 nonlinear regression analysis (Statistical Consultants, 1992) and the results for each group were averaged. The results are shown in Table 3.

TABLE 3

Comparison of IV Pharmacokinetic Parameters of N47
and r-HuEpo in Rats

Sample Test	Half-life		V_d (mL/kg)	Serum Clearance (mL/kg-hr)
	α (hours)	β (hours)		
N47 (n = 5 rats)	0.57±0.49	6.9±0.3	33±5	4.8±1.2
r-HuEpo (n = 6 rats)	0.18±0.03	2.5±0.2	36±6	17.7±3.4

a The results are presented as the group average \pm SD for five rats in the N47 group and six rats in the r-HuEpo group.

In the dog studies, normal Beagle dogs weighing between 7.8-9.5 kg received an intravenous bolus injection of ~29 μ Ci of either 125 I-rHuEpo or 125 I-N47 (~0.1 μ g peptide/kg) into the cephalic vein. At various time points through 24 hours post-administration, approximately 1 to 2 mL of blood was collected and serum prepared. The concentration of 125 I-rHuEpo and 125 I-N47 in 0.1mL serum was determined and pharmacokinetic parameters were calculated as described above. The serum concentration vs time pharmacokinetic curves for the dog studies are shown in Figure 5. The time points are the group means of two animals in each group. The pharmacokinetic parameters are summarized in Table 4.

TABLE 4

Comparison of IV Pharmacokinetic Parameters of N47
and r-HuEpo in Dogs

Sample Test	Half-life		V_d (mL/kg)	Serum Clearance (mL/kg-hr)
	α (hours)	β (hours)		
N47	0.34	25.0	55.9	2.4
r-HuEpo	0.40	7.2	60.8	8.4

a The results presented are the average parameters for the two dogs in each group.

In both the rat and dog studies, rHuEpo and Epo N47 analog exhibited a biphasic serum clearance. Clearance in rats was about 3.7-fold faster for rHuEpo than for Epo N47 analog and the β -half-life was about 2.8-fold longer for Epo N47 analog than for rHuEpo. The pharmacokinetic parameters in the dog studies were generally consistent with those observed in rat. In dogs, the clearance of rHuEpo was 3.5-fold faster than for Epo N47 analog and the β -half-life was 3.5-fold longer for Epo N47 analog compared with that for rHuEpo.

EXAMPLE 5

Dose response of hematocrit after administration of rHuEpo and Epo analog N47.

Hematocrit Dose Response Studies at three times per week (TIW)

The in vivo biological effects of rHuEpo and Epo analog N47 in normal mice were compared after administering a range of doses by either intraperitoneal or intravenous injection three times per week for up to six weeks. Hematocrit determinations were performed twice weekly by retro-orbital bleed.

Normal CD1 mice weighing approximately 30g (10-13 mice per group) were injected intraperitoneally three times per week for a total of six weeks with either rHuEpo (over the dose range of 0.625-10 μ g peptide/kg/dose), Epo N47 analog (over the dose range of 0.156-1.25 μ g peptide/kg/dose) or vehicle control. The vehicle control and diluent for the various rHuEpo and Epo N47 analog dosing preparations was phosphate-buffered saline (PBS),

containing 0.025% mouse serum albumin. The hematocrits of all mice were determined at baseline and twice weekly thereafter by retro-orbital bleeds. At the conclusion of the experiment, serum from all animals was collected and assayed for antibodies to the injected product by a solution radioimmunoprecipitation assay. Hematocrit data from animals judged to be negative for neutralizing antibodies were used for subsequent analysis.

As shown in Figure 6 both rHuEpo and Epo N47 analog produce a dose-dependent increase in hematocrit in the six week study, although N47 analog promotes a greater increase in hematocrit compared to rHuEpo at a given concentration. In this experiment the Epo N47 analog is about 3 to 4-fold more potent when dosed three times per week by intraperitoneal injection.

Dose response studies of rHuEpo and analog N47 were carried out by intravenous injection three times per week using procedures similar to those for intraperitoneal injection. The results obtained were similar to those for intraperitoneal administration and, in particular, the studies further confirmed that Epo N47 analog had a greater potency than rHuEpo when administered three times per week.

To better compare and quantify the biological activity of rHuEpo and Epo N47 analog in raising the hematocrit of normal mice, results of experiments were also analyzed by relative potency plots. For each experiment, the activity of rHuEpo or N47 analog at each dose was determined by summing the increase in hematocrit over the first 38 days of the study by trapezoidal summation to obtain the area under the curve (AUC). This was then plotted versus the log dose in μg peptide/kg/week. Potency difference between compounds administered by the same or different routes

of administration or dosing frequencies can be determined by measuring the distance between the relevant log-dose response lines. Figure 7 summarizes the relative potency data for all experiments performed comparing the activity of rHuEpo and Epo N47 analog administered by two different routes (intraperitoneal and intravenous) and at two different dosing schedules.

As shown in Figure 7 when administered three times per week, Epo N47 analog has the same potency when injected by either the intravenous or intraperitoneal route and was 3.6-fold more potent than rHuEpo injected intraperitoneally three times weekly.

Hematocrit Dose Response Studies at one time per week (OW)

Comparisons of rHuEpo and Epo analog N47 at increasing hematocrit in normal mice were undertaken with once weekly dosing by either the intraperitoneal or intravenous routes of administration for six weeks.

Normal CD1 mice weighing approximately 30g (8-10 mice per group) were injected intravenously once weekly for a total of six weeks with varying concentrations of either rHuEpo or Epo N47 analog prepared in PBS containing 0.025% mouse serum albumin, or with vehicle control (PBS with 0.025% mouse serum albumin). The analog dose varied from 6.25-25 µg of peptide/kg/dose and the dose of rHuEpo varied from 25-200 µg/kg/dose. The hematocrits of all mice were determined at baseline and twice weekly thereafter by retro-orbital bleeds. At the conclusion of the experiment, serum from all animals was collected and assayed for antibodies to the injected product by a solution radioimmunoprecipitation. Data from animals judged

to be negative for neutralizing antibodies were used for subsequent analysis.

As shown in Figure 8, whereas both rHuEpo and analog N47 can increase the hematocrit of normal mice when dosed once weekly, the dose of rHuEpo required to produce a response was significantly greater than that for analog N47. For instance, in this experiment 25µg peptide/kg/week of N47 increased the hematocrit of mice by 41.2 points in six weeks, whereas the same dose of rHuEpo produced only a 12.5 point hematocrit rise.

Dose response studies of rHuEpo and analog N47 were performed by intraperitoneal injection once weekly using procedures similar to those described above. The results obtained were consistent with the results for intravenous administration and further confirmed the greater potency of analog N47 compared with rHuEpo when administered one time per week.

To quantify the activity difference between rHuEpo and N47 analog when each is dosed once weekly, relative potency plots were generated from all relevant experiments as described above. As shown in Figure 7, when administered one time per week, analog N47 has the same potency when injected by the intravenous and intraperitoneal route. Analog N47 is approximately 14-fold more potent than rHuEpo when each is administered once weekly.

In addition, the log-dose response plots in Figure 6 also illustrate the following: (1) A given dose of analog N47 administered once weekly (QW) is approximately as effective as the same total weekly dose of rHuEpo given as three divided doses (TIW); (2) A given dose of rHuEpo administered once weekly (QW) is only approximately 2% as effective as the same total weekly dose of analog N47 given as three divided doses

(TIW); (3) Analog N47 is approximately 4-fold more potent in mice when administered TIW compared to QW.

Hematocrit Dose Response Studies at Every Other Week
5 (EOW)

Experiments were also undertaken to assess the ability of analog N47 to increase the hematocrit of mice when injected once every other week. Normal CD-1 mice (10 mice per group) were injected intravenously
10 either once weekly or once every other week for a total of approximately six weeks with varying concentrations of Epo N47 analog prepared in PBS containing 0.025% mouse serum albumin. Analog N47 was administered at
15 either 200, 100 or 25µg/kg/dose every other week or at 12.5µg/kg/dose once weekly. The hematocrits of all mice were determined at baseline and twice weekly thereafter by retroorbital bleeds.

As shown in Figure 9, analog N47 can increase the hematocrit of normal mice in a dose-dependent
20 fashion even when administered bi-monthly. As expected when dosed less frequently, a greater amount of N47 analog is required to increase the hematocrit. A dose of 200 µg/kg of N47 analog administered every other week increased the hematocrit to approximately the same
25 extent in six weeks as did 12.5 µg/kg when dosed weekly.

EXAMPLE 6

IV Pharmacokinetics of Epo N47 analog
30 and rHuEpo in Continuous Ambulatory Peritoneal Dialysis
(CAPD) patients

In view of the marked increase in serum half-life of Epo N47 analog compared to rHuEpo in rat

and beagle dog, it was of interest to determine whether an increase could also be observed in humans.

A double-blind, randomized cross-over design study of eleven stable CAPD patients (7 males, 4 females, aged 27-75 years) was undertaken. One patient group received 100U/kg of rHuEpo (equivalent to 0.5 μ g of peptide/kg) while a second group of patients received 0.5 μ g peptide/kg of Epo N47 analog, both administered as a single bolus injection intravenous. Venous blood samples (3mL) were drawn via an indwelling cannula and were taken pre-dose and at 5, 10, 15, 30 minutes and 1, 2, 5, 8, 12, 16, 24, 30, 36, 48, 60, 72 and 96 hours after the intravenous bolus. After a 28 day washout period, the first patient group received a single intravenous dose of Epo analog N47 while the second group received a single intravenous dose of rHuEpo. Blood samples were taken as in the first cycle of treatment. Levels of rHuEpo and Epo N47 analog were determined in serum by ELISA after subtraction of baseline endogenous Epo levels. The pharmacokinetic parameters (mean \pm SE) estimated after adjustment for cross-over design effects are shown in Table 5. The serum concentration AUC was calculated using the linear trapezoidal summation. $t_{1/2_z}$ is defined as: $\log(2)/K_z$, where K_z is calculated as the slope of the terminal portion of the \ln (serum concentration) time curve. The clearance (Cl) is defined as: dose/AUC. The volume of distribution (V_d) is defined as: Cl/K .

30

TABLE 5

Dose Group	AUC (ng.h/mL)	$t_{1/2_z}$ (h)	Cl (mL/h/kg)	V_d (mL/kg)
N47	291.0 \pm 7.6	25.3 \pm 2.2	1.6 \pm 0.3	52.4 \pm 2.0
rHuEpo	131.9 \pm 8.3	8.5 \pm 2.4	4.0 \pm 0.3	48.7 \pm 2.1

The mean serum half-life for Epo N47 analog (25.3 hr) was three times longer than for rHuEpo (8.5 hr) and the clearance was 2.5-fold faster for rHuEpo than for analog N47.

EXAMPLE 7

10 A Phase II Dose Finding and Dose Scheduling
Study of Epo N47 Analog

Multicenter, randomized, sequential dose-escalation studies are initiated to investigate the optimum dose and dose schedule for analog N47 when administered by subcutaneous or intravenous injection in patients with CRF receiving dialysis.

The dosing schedule is as follows:

20 Once per week dosing: 0.075, 0.225, 0.45, 0.75, 1.5 and 4.5 µg of peptide/kg/dose.

30 Three times per week dosing: 0.025, 0.075, 0.15, 0.25, 0.5 and 1.5 µg of peptide/kg/dose.

The studies are carried out in two parts: the first part is a dose-escalation study designed to evaluate the dose of analog N47 given either one or three times per week which increases hemoglobin at an optimum rate over four weeks (greater than or equal to 1 g/dL but less than 3 g/dL). The second part of each study is designed to determine the doses required (when administered once or three times per week by either the intravenous or subcutaneous routes of administration) to maintain the hematocrit at the therapeutic target.

35 Preliminary results indicate that once weekly dosing with analog N47 can be used to both

increase and maintain the hematocrit of anemic CRF patients. Initial results suggest that the preferred doses to initiate therapy on a three times a week dosing schedule are 0.15 and 0.25

5 $\mu\text{g}/\text{peptide}/\text{kg}/\text{dose}$, and on a one time per week dosing schedule are 0.45 and 0.75 $\mu\text{g}/\text{peptide}/\text{kg}/\text{dose}$ for both routes of administration.

* * *

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While the invention has been described in what is considered to be its preferred embodiments, it is not to be limited to the disclosed embodiments, but on the contrary, is intended to
15 cover various modifications and equivalents included within the spirit and scope of the appended claims, which scope is to be accorded the broadest interpretation so as to encompass all such
20 modifications and equivalents.

WHAT IS CLAIMED IS:

1. A method of raising and maintaining hematocrit in a mammal comprising administering a therapeutically effective amount of a hyperglycosylated analog of erythropoietin in a pharmaceutical composition, wherein the analog is administered less frequently than an equivalent molar amount of recombinant human erythropoietin to obtain a comparable target hematocrit.
2. The method of Claim 1 wherein the amount of hyperglycosylated analog of erythropoietin is administered about two times per week.
3. The method of Claim 1 wherein the amount of hyperglycosylated analog of erythropoietin is administered about one time per week.
4. The method of Claim 1 wherein the amount of hyperglycosylated analog of erythropoietin is administered about one time every other week.
5. The method of Claim 1 wherein the amount of hyperglycosylated analog of erythropoietin is administered about one time per month.
6. The method of Claim 3 wherein the amount of hyperglycosylated analog of erythropoietin that is administered is about 0.075 to 4.5 μg per erythropoietin peptide per kg per dose.
7. A method of raising and maintaining hematocrit in a mammal comprising administering a therapeutically effective amount of a hyperglycosylated

analog of erythropoietin in a pharmaceutical composition, wherein the analog is administered at a lower molar amount than recombinant human erythropoietin to obtain a comparable target hematocrit.

8. The method of Claim 7 whereas the amount of hyperglycosylated analog of erythropoietin that is administered is about 0.025 to 1.5 µg erythropoietin peptide per kg per dose three times per week.

9. The method of Claims 1 or 7 wherein the hyperglycosylated analog of erythropoietin comprises at least one additional glycosylation site compared to human erythropoietin wherein a carbohydrate chain is added to the site.

10. The method of Claims 1 or 7 wherein the target hematocrit is at least about 30%.

11. The analog of Claim 9 wherein the carbohydrate chain is an N-linked carbohydrate chain at one or more of positions 30, 51, 57, 69, 88, 89, 136 and 138 of the sequence of human erythropoietin.

12. The analog of Claim 9 which has additional N-linked carbohydrate chains at position 30 and 88 of the sequence of human erythropoietin.

13. The analog of Claim 12 which is Asn³⁰Thr³²Val⁸⁷Asn⁸⁸Thr⁹⁰Epo.

14. The method of Claims 1 or 7 wherein the pharmaceutical composition comprises a pharmaceutically

acceptable diluent, carrier, solubilizer, emulsifier, preservative, and/or adjuvant.

15 15. The composition of Claim 14 wherein the diluent is a buffer solution of sodium citrate or sodium phosphate.

10 16. The composition of Claim 14 wherein the carrier is human serum albumin.

17. The composition of Claim 14 wherein the preservative is benzyl alcohol.

15 18. The method of Claims 1 or 7 wherein the mammal suffers from anemia associated with a decline or loss of kidney function.

20 19. The method of Claims 1 or 7 wherein the mammal suffers from anemia associated with myelosuppressive therapy.

25 20. The method of Claim 19 wherein the myelosuppressive therapy comprises chemotherapeutic or anti-viral drugs.

21. The method of Claims 1 or 7 wherein the mammal suffers from anemia associated with excessive blood loss.

30 22. The method of Claims 1 or 7 further comprising administering a therapeutically effective amount of iron.

35 23. An analog of human erythropoietin comprising at least one additional glycosylation site at any of positions 52, 53, 55, 86 and 114 of the

sequence of human erythropoietin, wherein an N-linked carbohydrate chain is added to the site.

24. The analog of Claim 22 comprising at least two additional glycosylation sites wherein a carbohydrate chain is attached to each of the sites.

25. The analog of Claim 22 comprising at least three additional glycosylation sites wherein a carbohydrate chain is attached to each of the sites.

26. The analog of Claim 22 comprising at least four additional glycosylation sites wherein a carbohydrate chain is attached to each of the sites.

27. An analog of human erythropoietin selected from the group consisting of:

Asn⁵² Thr⁵⁴ Epo;
Asn⁵³ Thr⁵⁵ Epo;
Asn³⁰ Thr³² Val⁸⁷ Asn⁸⁸ Thr⁹⁰ Thr¹²⁵ Epo;
Asn¹¹⁴ Thr¹¹⁶ Epo;
Asn³⁰ Thr³² Asn⁵³ Thr⁵⁵ Val⁸⁷ Asn⁸⁸ Thr⁹⁰ Epo;
Asn⁵⁵ Thr⁵⁷ Epo;
Asn⁸⁶ Val⁸⁷ Thr⁸⁸ Epo;
Ala⁸⁷ Asn⁸⁸ Thr⁹⁰ Epo;
Val⁸⁷ Asn⁸⁸ Ser⁹⁰ Epo;
Val⁸⁷ Asn⁸⁸ Gly⁸⁹ Thr⁹⁰ Epo;
Asn³⁰ Thr³² Asn⁵³ Thr⁵⁵ Epo;
Asn³⁰ Thr³² Asn¹¹⁴ Thr¹¹⁶ Epo; and
Asn³⁰ Thr³² Asn⁵³ Thr⁵⁵ Val⁸⁷ Asn⁸⁸ Thr⁹⁰ Asn¹¹⁴ Thr¹¹⁶ Epo.

28. The analog of Claims 23-27 which is the product of expression of an exogenous DNA sequence.

29. A DNA sequence encoding the analog of Claims 23-27.

30. A eucaryotic host cell transfected with the DNA sequence of Claim 29 in a manner allowing the host cell to express the analog.

31. A composition comprising a therapeutically effective amount of the analog of Claims 23-27 together with a pharmaceutically acceptable diluent, adjuvant or carrier.

32. A method of raising and maintaining hematocrit in a mammal comprising administering a therapeutically effective amount of the analog of Claims 23-27 in a pharmaceutical composition.

33. The method of Claim 32 wherein the analog is administered less frequently than an equivalent molar amount of recombinant human erythropoietin to obtain a comparable target hematocrit.

34. The method of Claim 33 wherein the amount of analog is administered about one time per week, about one time every other week, or about one time per month.

35. The method of Claim 32 wherein the analog is administered at a lower molar amount than recombinant human erythropoietin to obtain a comparable target hematocrit.

36. The method of Claim 34 wherein the amount of analog that is administered is about 0.025 to

1.5 µg erythropoietin peptide per kg per dose three times per week.

37. The method of Claim 34 wherein the amount of analog that is administered is less than about 0.025 µg erythropoietin peptide per kg per dose three times per week.

38. A fusion protein comprising a hyperglycosylated analog of erythropoietin and an immunoglobulin heavy chain constant region.

39. The fusion protein of claim 38 wherein the immunoglobulin heavy chain constant region is an Fc region.

40. The fusion protein of Claim 39 wherein the Fc region is from human IgG or derived from human IgG.

41. The fusion protein of Claim 40 wherein the IgG is IgG1, IgG2, IgG3 or IgG4, or a combination thereof.

42. The fusion protein of Claim 38 comprising at least one additional glycosylation site at any of positions 30, 51, 52, 53, 55, 57, 69, 86, 88, 89, 114, 136 and 138 of the sequence of human erythropoietin, wherein an N-linked carbohydrate chain is added at the site.

43. The fusion protein of Claim 38 which is Asn³⁰Thr³²Val⁸⁷Asn⁸⁸Thr⁹⁰ Epo fused to an Fc region.

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[illegible]

ABSTRACT

5 Methods for increasing and maintaining
hematocrit in a mammal comprising administering a
hyperglycosylated analog of erythropoietin are
disclosed. An analog may be administered less
frequently than an equivalent molar amount of
10 recombinant human erythropoietin to obtain a comparable
target hematocrit and treat anemia. Alternatively, a
lower molar amount of a hyperglycosylated analog may be
administered to obtain a comparable target hematocrit
and treat anemia. Also disclosed are new
15 hyperglycosylated erythropoietin analogs, methods of
production of the analogs, and compositions comprising
the analogs.

FIG.1

Met Gly Val His Glu Cys Pro Ala Trp Leu Trp Leu Leu Leu Ser Leu
-25 -20 -15

Leu Ser Leu Pro Leu Gly Leu Pro Val Leu Gly Ala Pro Pro Arg Leu
-10 -5 1 5

Ile Cys Asp Ser Arg Val Leu Glu Arg Tyr Leu Leu Glu Ala Lys Glu
10 15 20

Ala Glu Asn Ile Thr Thr Gly Cys Ala Glu His Cys Ser Leu Asn Glu
25 30 35

Asn Ile Thr Val Pro Asp Thr Lys Val Asn Phe Tyr Ala Trp Lys Arg
40 45 50

Met Glu Val Gly Gln Gln Ala Val Glu Val Trp Gln Gly Leu Ala Leu
55 60 65

Leu Ser Glu Ala Val Leu Arg Gly Gln Ala Leu Leu Val Asn Ser Ser
70 75 80 85

Gln Pro Trp Glu Pro Leu Gln Leu His Val Asp Lys Ala Val Ser Gly
90 95 100

Leu Arg Ser Leu Thr Thr Leu Leu Arg Ala Leu Gly Ala Gln Lys Glu
105 110 115

Ala Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala Pro Leu Arg Thr Ile
120 125 130

Thr Ala Asp Thr Phe Arg Lys Leu Phe Arg Val Tyr Ser Asn Phe Leu
135 140 145

Arg Gly Lys Leu Lys Leu Tyr Thr Gly Glu Ala Cys Arg Thr Gly Asp
150 155 160 165

Arg

FIG. 2

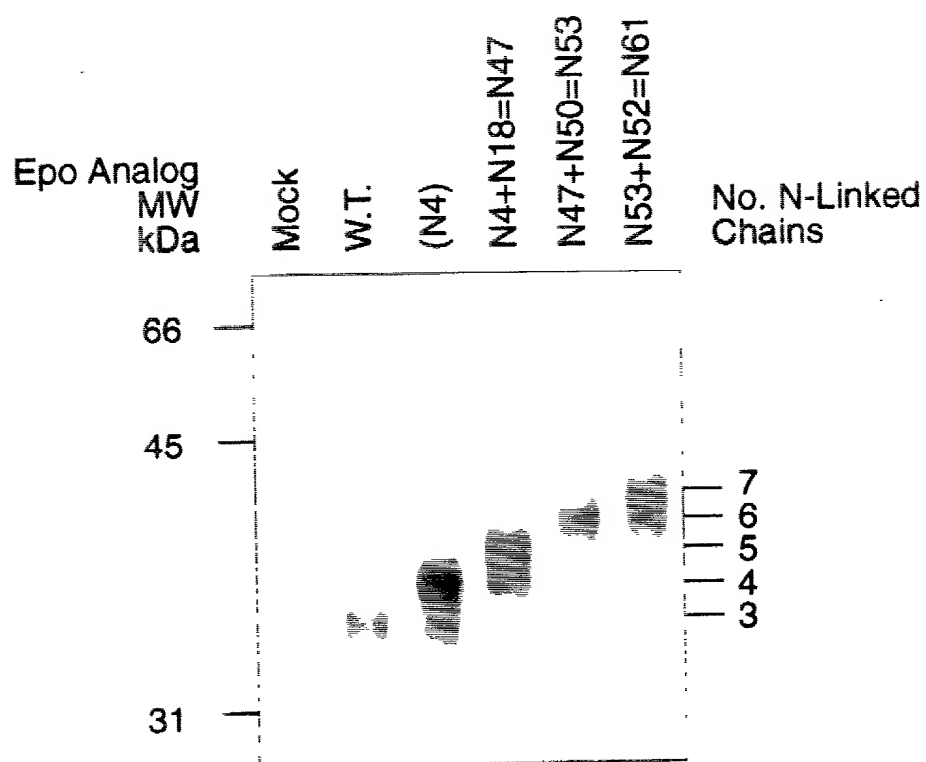
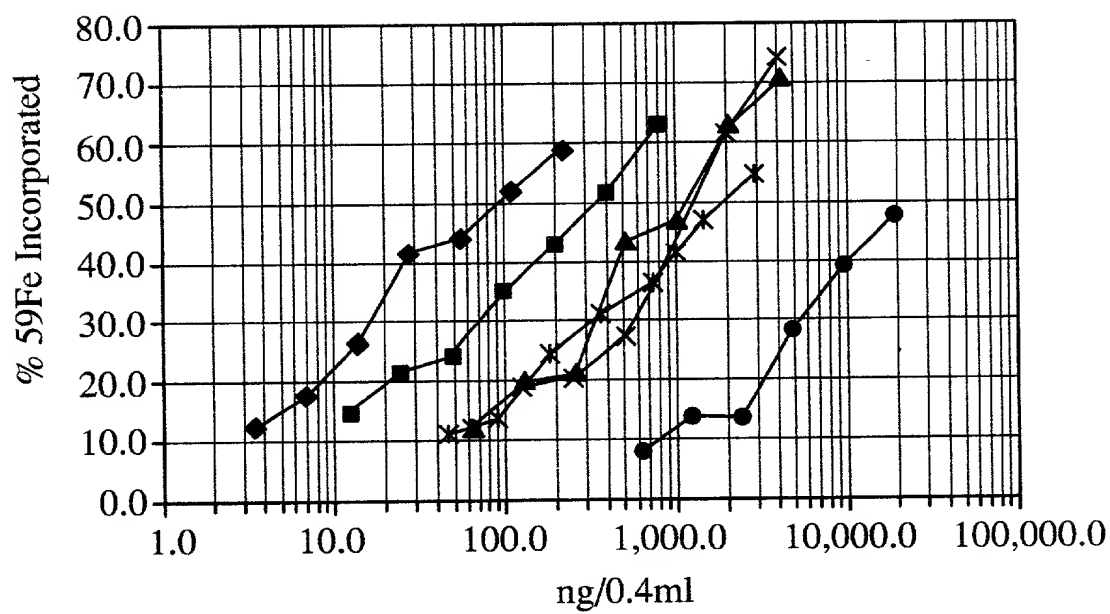


FIG. 3



- ◆ N53
- ▲ N4
- × N18
- rHuEPO
- N47
- * N50

FIG.4

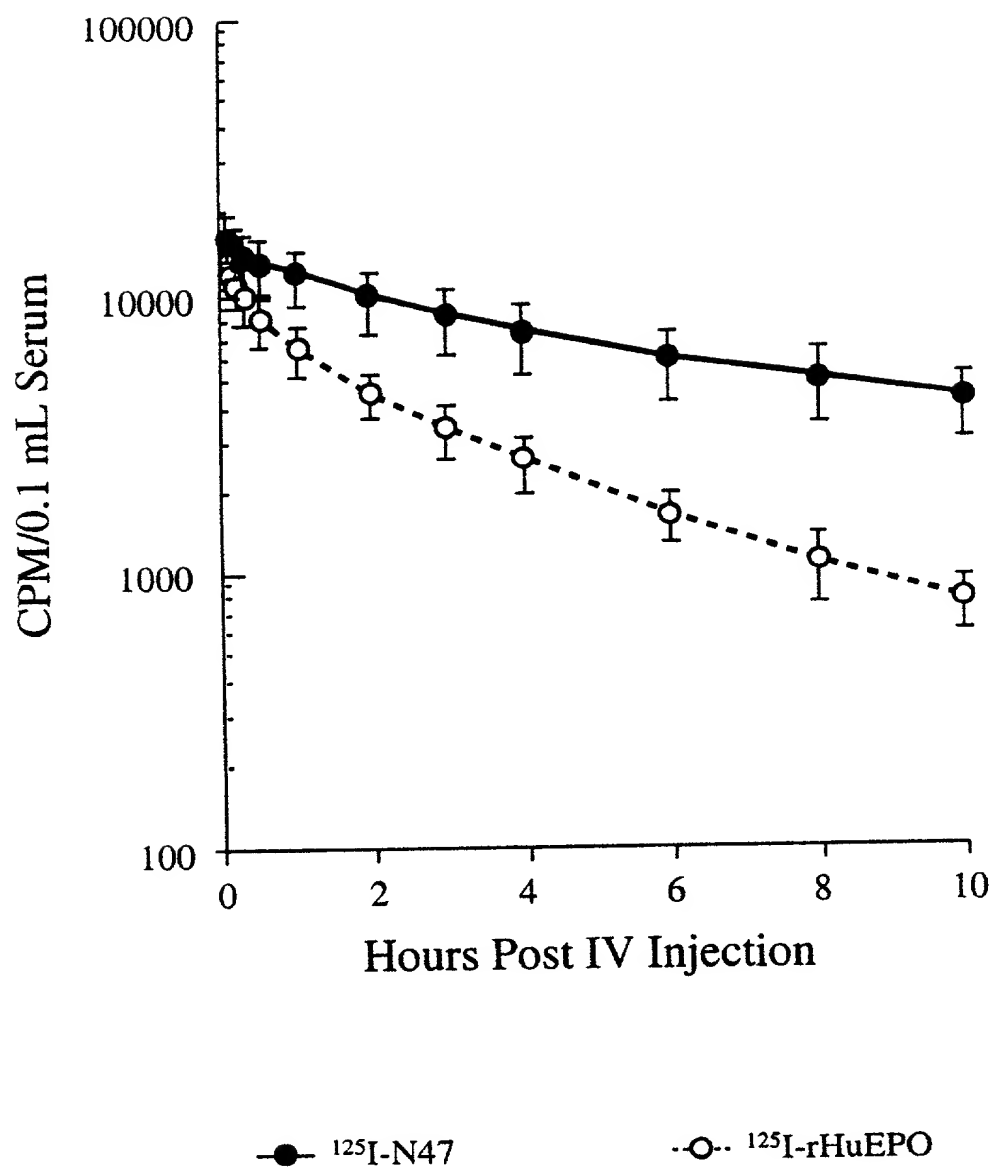


FIG.5

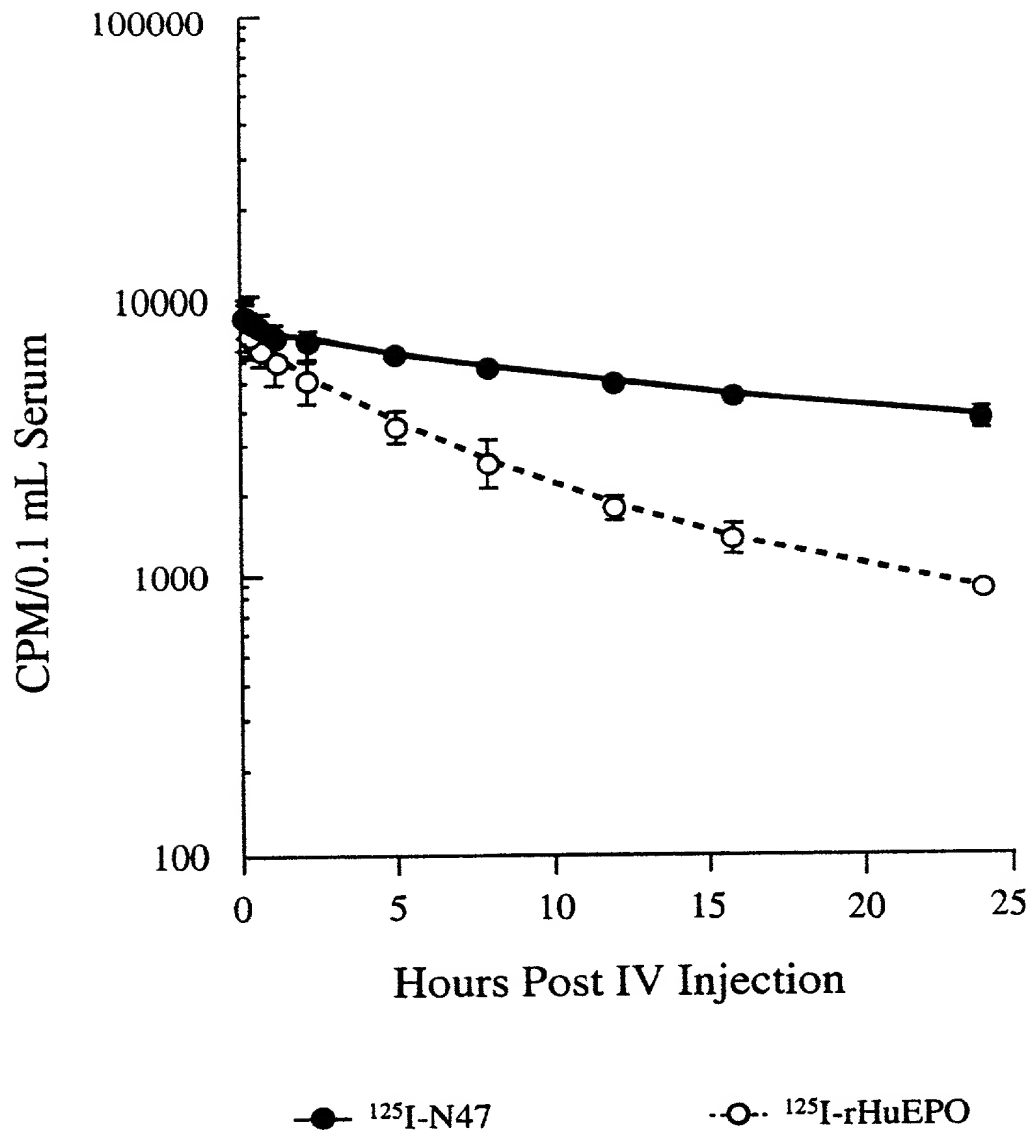
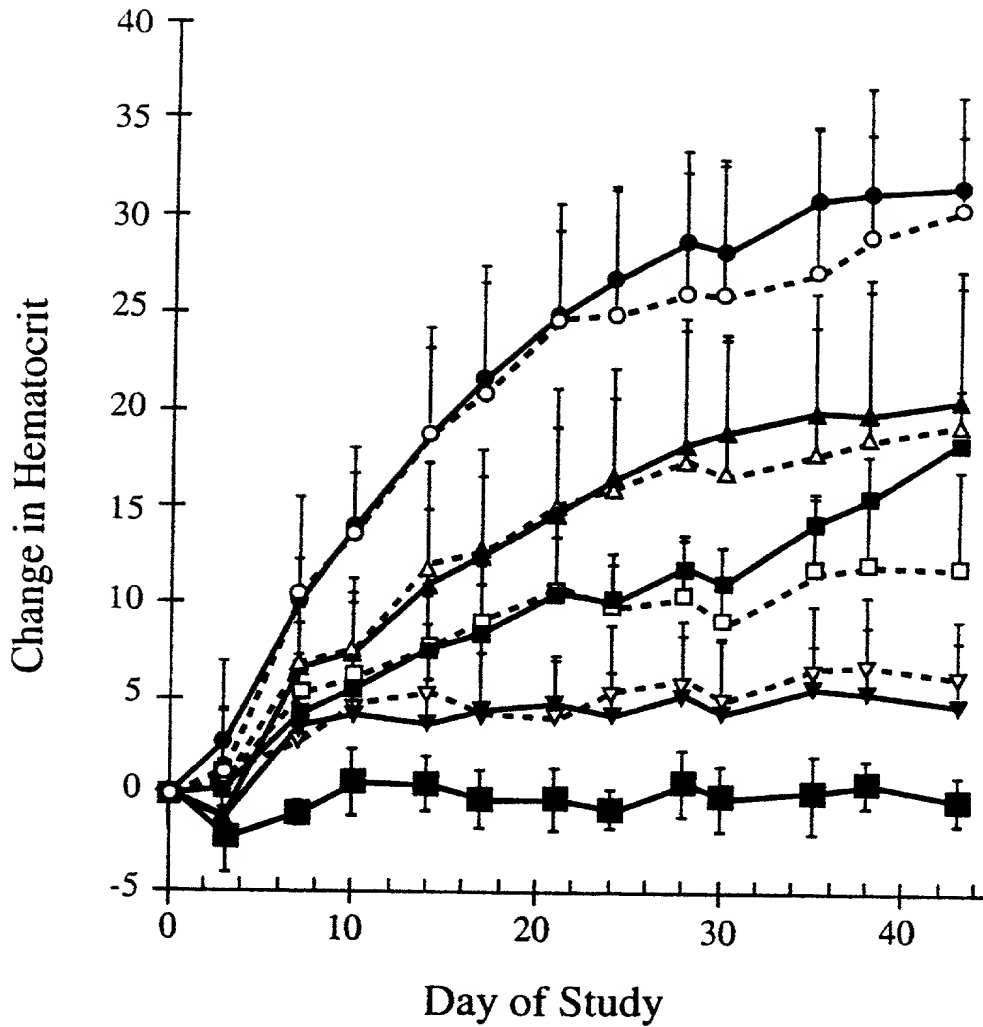
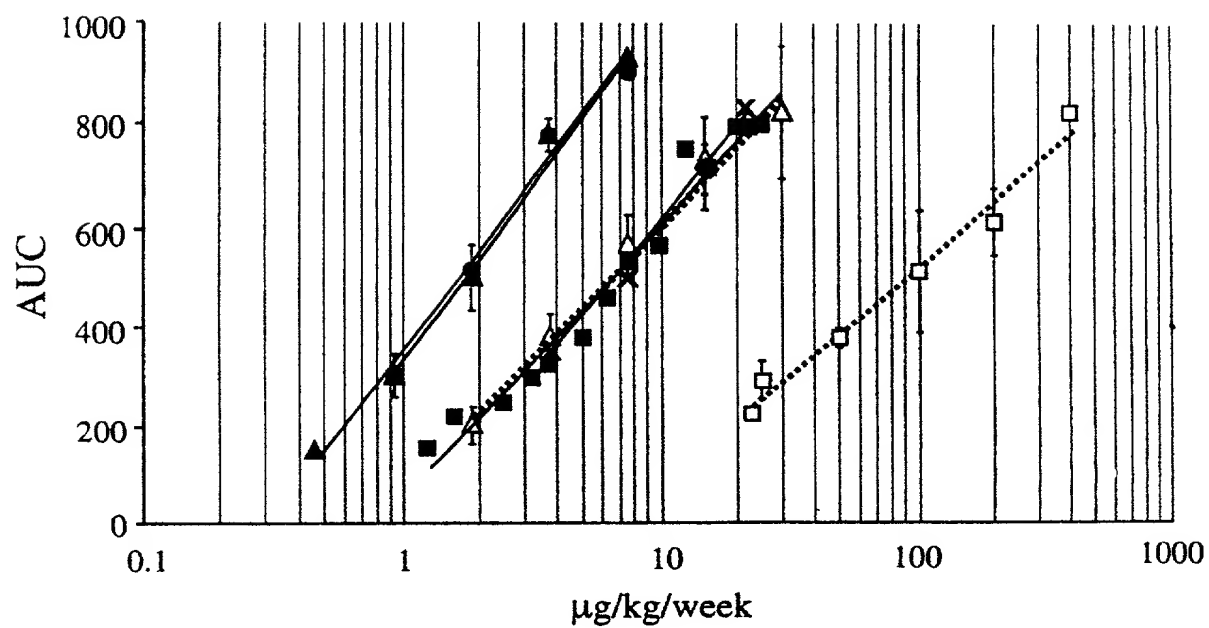


FIG.6



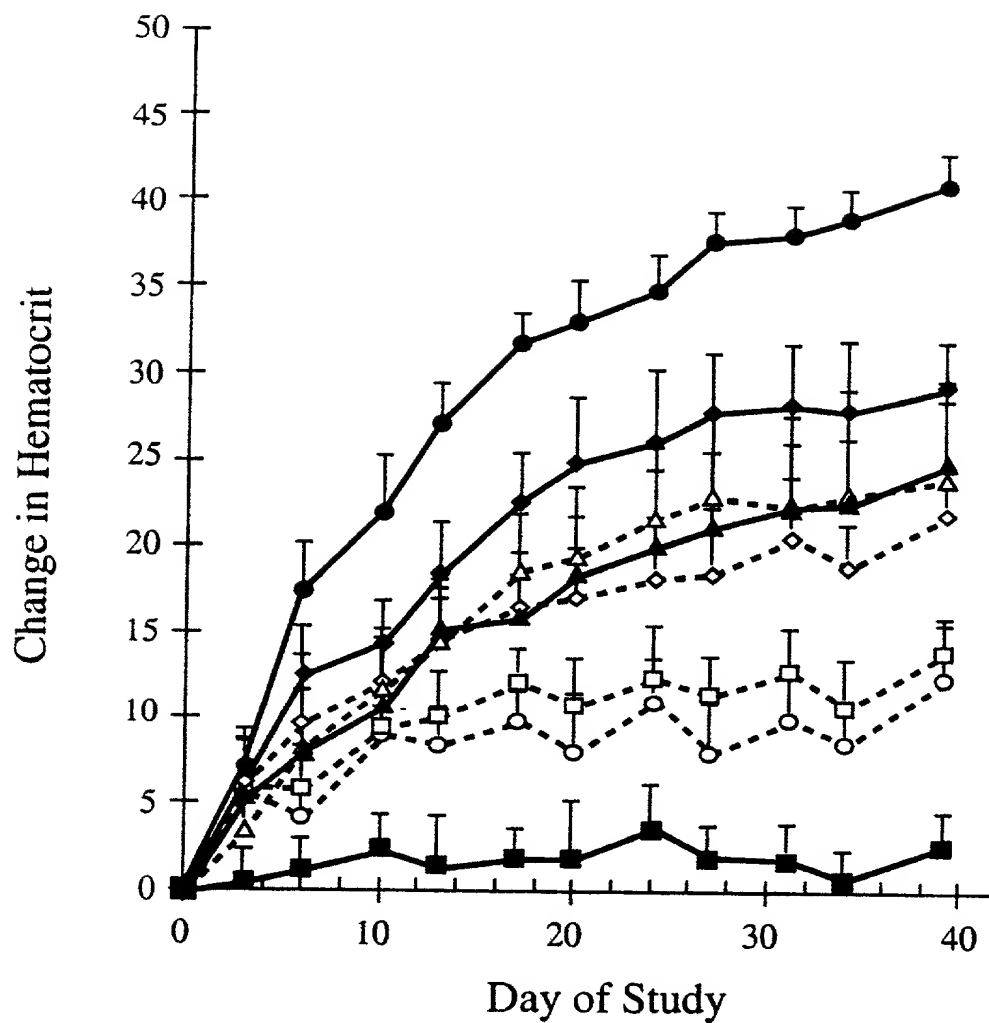
- N47 1.25 µg/kg/dose (IP-TIW)
- ▲ N47 0.625 µg/kg/dose (IP-TIW)
- N47 0.313 µg/kg/dose (IP-TIW)
- ▼ N47 0.156 µg/kg/dose (IP-TIW)
- vehicle (IV-TIW)
- rHuEPO 10 µg/kg/dose (IP-TIW)
- △ rHuEPO 2.5 µg/kg/dose (IP-TIW)
- rHuEPO 1.25 µg/kg/dose (IP-TIW)
- ▽ rHuEPO 0.625 µg/kg/dose (IP-TIW)

FIG.7



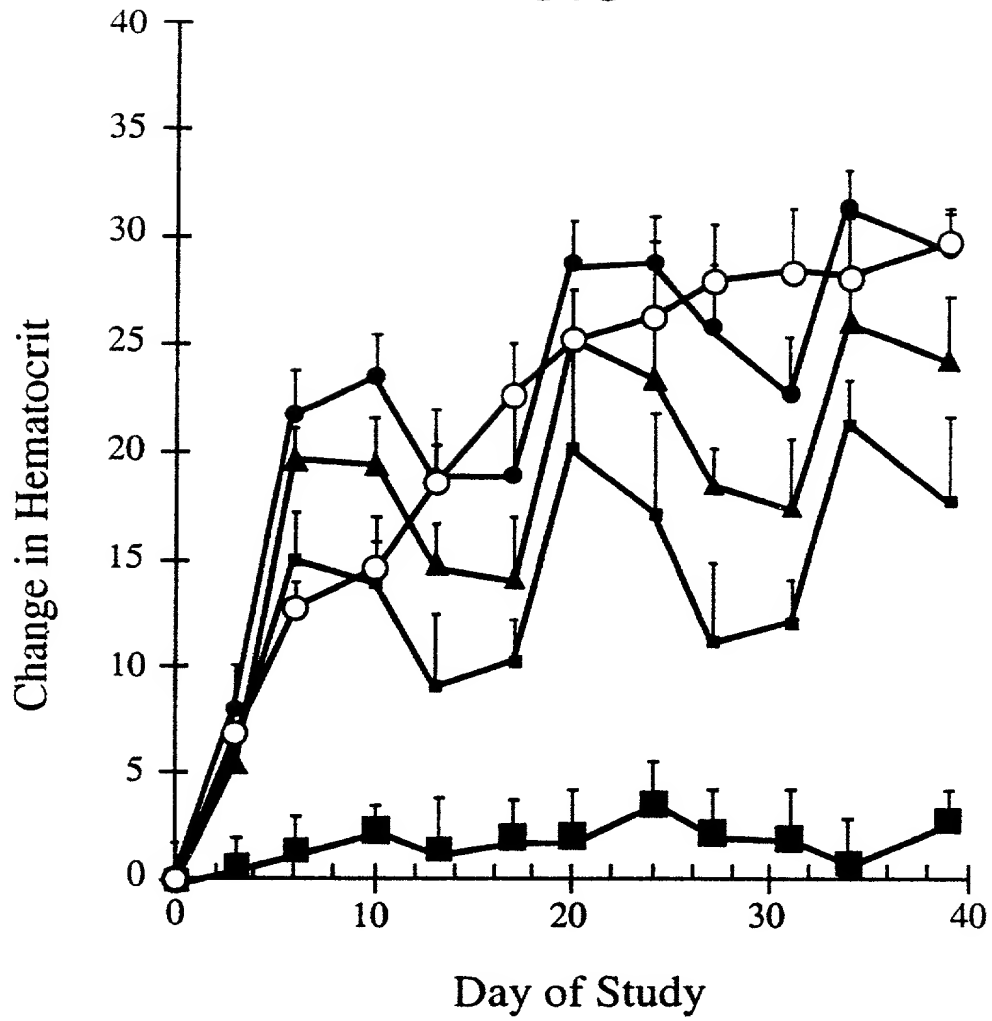
- N47 (IV TIW)
- ▲ N47 (IP TIW)
- N47 (IV QW)
- × N47 (IP QW)
- △ rHuEPO (IP TIW)
- rHuEPO (IV QW)

FIG.8



- N47 25 µg/kg/dose (IV-QW)
- ◆ N47 12.5 µg/kg/dose (IV-QW)
- ▲ N47 6.25 µg/kg/dose (IV-QW)
- vehicle (IV-QW)
- △ rHuEPO 200 µg/kg/dose (IV-QW)
- ◇ rHuEPO 100 µg/kg/dose (IV-QW)
- rHuEPO 50 µg/kg/dose (IV-QW)
- rHuEPO 25 µg/kg/dose (IV-QW)

FIG.9



- N47 200 µg/kg/dose (IV-EOW)
- ▲ N47 100 µg/kg/dose (IV-EOW)
- N47 25 µg/kg/dose (IV-EOW)
- N47 12.5 µg/kg/dose (IV-QW)
- vehicle

Figure 1 shows a schematic representation of 12 genes and their corresponding cDNA clones. The genes are arranged in two columns. The left column contains genes 1 through 12, and the right column contains genes 1 through 12. The cDNA clones are shown as horizontal bars with their corresponding gene names. The genes are: 1. 5S rRNA, 2. 18S rRNA, 3. 28S rRNA, 4. 5.8S rRNA, 5. 5.8S rRNA, 6. 5.8S rRNA, 7. 5.8S rRNA, 8. 5.8S rRNA, 9. 5.8S rRNA, 10. 5.8S rRNA, 11. 5.8S rRNA, 12. 5.8S rRNA. The cDNA clones are: 1. 5S rRNA, 2. 18S rRNA, 3. 28S rRNA, 4. 5.8S rRNA, 5. 5.8S rRNA, 6. 5.8S rRNA, 7. 5.8S rRNA, 8. 5.8S rRNA, 9. 5.8S rRNA, 10. 5.8S rRNA, 11. 5.8S rRNA, 12. 5.8S rRNA.

[illegible]

1 aagcttcttagaccaccatggtgggtgcacgaatgtcctgcctggctgtggcttctcctgtc 60
-----+-----+-----+-----+-----+
ttcgaagatctggtggtacccccacgtgcttacaggacggaccgacaccgaagaggacag
M G V H E C P A W L W L L L S -
61 cctgtctgtcgtccctctggtggcctcccagtcctgggcGCCCCACCACGCCTCATCTGTGA 120
-----+-----+-----+-----+-----+
ggacgacagcgaggggagacccggagggtcaggaccccgCGGGGTGGTGC GGAGTAGACACT
b L L S L P L G L P V L G A P P R L I C D -
121 CAGCCGAGTCCCTGGAGAGGTACCTCTTGGAGGCCAAGGAGGCCGAGAATATCACGACGGG 180
-----+-----+-----+-----+-----+
GTCGGCTCAGGACCTCTCCATGGAGAACCTCCGGTTCTCCTCCGGCTCTTATAGTGCTGCCC
b S R V L E R Y L L E A K E A E N I T T G -
181 CTGTaatGAAacgTGCAGCTTGAATGAGAATATCACTGTCCCAGACACCAAAGTTAATTT 240
-----+-----+-----+-----+-----+
GACAttaCTTtgCACGTCGAACTTACTCTTATAGTGACAGGGTCTGTGGTTTCAATTAAA
b C N E T C S L N E N I T V P D T K V N F -
241 CTATGCCTGGAAGAGGATGGAGGTCTGGGCAGCAGGCCGTAGAAGTCTGGCAGGGCCTGGC 300
-----+-----+-----+-----+-----+
GATACGGACCTTCTCCTACCTCCAGCCCGTCGTCCGGCATCTTCAGACCGTCCCGGACCG
b Y A W K R M E V G Q Q A V E V W Q G L A -
301 CCTGCTGTCTGGAAGCTGTCTCTGCGGGGCCAGGCCCTGTTGGTCAACTCTTCCCAGgtgaa 360
-----+-----+-----+-----+-----+
GGACGACAGCCTTCGACAGGACGCCCCGGTCCGGGACAACCAGTTGAGAAGGGTCcactt
b L L S E A V L R G Q A L L V N S S Q V N -
361 tGAGaCCCTGCAGCTGCATGTGGATAAAGCCGTCAGTGGCCTTCGCAGCCTCACCACTCT 420
-----+-----+-----+-----+-----+
aCTCtGGGACGTCGACGTACACCTATTTTCGGCAGTCACCGGAAGCGTCGGAGTGGTGAGA
b E T L Q L H V D K A V S G L R S L T T L -
421 GCTTCGGGCTCTGGGAGCCCAGAAGGAAGCCATCTCCCCCTCCAGATGCGGCCTCAGCTGC 480
-----+-----+-----+-----+-----+
CGAAGCCCCGAGACCCTCGGGTCTTCCTTCGGTAGAGGGGAGGTCTACGCCGGAGTCGACG
b L R A L G A Q K E A I S P P D A A S A A -
481 TCCACTCCGAACAATCACTGCTGACACTTTCCGCAAACCTCTTCCGAGTCTACTCCAATTT 540
-----+-----+-----+-----+-----+
AGGTGAGGCTTGTTAGTGACGACTGTGAAAGGCGTTTGAGAAGGCTCAGATGAGGTTAAA
b P L R T I T A D T F R K L F R V Y S N F -
541 CCTCCGGGGAAAGCTGAAGCTGTACACAGGGGAGGCCTGCAGGACAGGGGACAGAGACAA 600
-----+-----+-----+-----+-----+
GGAGGCCCCCTTTCGACTTCGACATGTGTCCCCTCCGGACGTCTGTCCCCTGTCTCTGTT
b L R G K L K L Y T G E A C R T G D R D K -
601 AACTCACACATGTCCACCTTGTCCAGCTCCGGAACCTCTGGGGGGTCTTTCAGTCTTCCT 660
-----+-----+-----+-----+-----+
TTGAGTGTGTACAGGTGGAACAGGTTCGAGGCTTGAGGACCCCCCAGGAAGTCAGAAGGA
b T H T C P P C P A P E L L G G P S V F L -

FIG. 11, cont.

```

CTTCCCCC AAAACCCAAGGACACCCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGT
661 -----+-----+-----+-----+-----+-----+-----+ 720
GAAGGGGGGTTTGGGTTTCCTGTGGGAGTACTAGAGGGCCTGGGGACTCCAGTGTACGCA

b      F P P K P K D T L M I S R T P E V T C V -

GGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGT
721 -----+-----+-----+-----+-----+-----+-----+ 780
CCACCACCTGCACTCGGTGCTTCTGGGACTCCAGTTCAAGTTGACCATGCACCTGCCGCA

b      V V D V S H E D P E V K F N W Y V D G V -

GGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGT
781 -----+-----+-----+-----+-----+-----+-----+ 840
CCTCCACGTATTACGGTTCTGTTTCGGCGCCCTCCTCGTCATGTTGTCGTGCATGGCACA

b      E V H N A K T K P R E E Q Y N S T Y R V -

GGTCAGCGTCTCTACCGTCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAA
841 -----+-----+-----+-----+-----+-----+-----+ 900
CCAGTCGCAGGAGTGGCAGGACGTGGTCTTGACCGACTTACCGTTCTCATGTTACGTT

b      V S V L T V L H Q D W L N G K E Y K C K -

GGTCTCCAACAAAGCCCTCCCAGCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCA
901 -----+-----+-----+-----+-----+-----+-----+ 960
CCAGAGGTTGTTTCGGGAGGGTCGGGGGTAGCTCTTTTGGTAGAGGTTTCGGTTTCCCGT

b      V S N K A L P A P I E K T I S K A K G Q -

GCCCCGAGAACCACAGGTGTACACCCTGCCCCATCCCGGGATGAGCTGACCAAGAACCA
961 -----+-----+-----+-----+-----+-----+-----+ 1020
CGGGGCTCTTGGTGTCCACATGTGGGACGGGGTAGGGCCCTACTCGACTGGTTCTTGGT

b      P R E P Q V Y T L P P S R D E L T K N Q -

GGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGA
1021 -----+-----+-----+-----+-----+-----+-----+ 1080
CCAGTCGGACTGGACGGACCAAGTTTCCGAAGATAGGGTCGCTGTAGCGGCACCTCACCTT

b      V S L T C L V K G F Y P S D I A V E W E -

GAGCAATGGGCAGCCGGAACAACATAAGACCACGCCTCCCGTGCCTGGACTCCGACGG
1081 -----+-----+-----+-----+-----+-----+-----+ 1140
CTCGTTACCCGTCGGCCTCTTGTGATGTTCTGGTGGGAGGGACGACCTGAGGCTGCC

b      S N G Q P E N N Y K T T P P V L D S D G -

CTCCTTCTTCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGT
1141 -----+-----+-----+-----+-----+-----+-----+ 1200
GAGGAAGAAGGAGATGTCGTTTCGAGTGGCACCTGTTCTCGTCCACCGTCGTCCCCTTGCA

      S F F L Y S K L T V D K S R W Q Q G N V -

CTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTC
1201 -----+-----+-----+-----+-----+-----+-----+ 1260
GAAGAGTACGAGGCACTACGTACTCCGAGACGTGTTGGTGATGTGCGTCTTCTCGGAGAG

      F S C S V M H E A L H N H Y T Q K S L S -

                                SalI
                                |
CCTGTCTCCGGGTAAAtaatgtcgac
1261 -----+-----+-----+-----+-----+-----+ 1286
GGACAGAGGCCCATTTattacagctg

      L S P G K *

```

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first, and sole inventor (if only one name is listed below) or a joint inventor (if plural names are listed below) of the invention entitled

METHODS AND COMPOSITIONS FOR THE PREVENTION AND TREATMENT OF ANEMIA

which is described and claimed in the specification which:

- ☒ is attached hereto.
☐ was filed on _____
 as Application Serial No. _____
 and was amended on _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1.56(a).

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the filing date of this application:

APPLICATION SERIAL NO.	FILING DATE	STATUS
09/178,292	October 23, 1998	Pending

Power of Attorney: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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